DEVELOPMENT OF NOVEL METHODS FOR THE DETECTION OF COUMARIN, AND ITS METABOLITES, AND THEIR APPLICATIONS

A dissertation submitted for the degree of

Doctor of Philosophy

by

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Is it I, or is it not !

It means nothing

Yet it explains the world

It achieves so little

Yet does so much

It can't go on

Yet it fails to be stopped

It grows, multiplies, progresses

Yet it never changes

It concentrates on a void

Yet produces a masterpiece

It is darkness

Yet it lights up in us

It is nothing

But it is something

It is death and life

Yet it contradicts itself

It is a paradox, an enigma

Yet fully comprehensible

It is I

And it is not

March 8th, 1991, Declan P Bogan

DECLARATION

I hereby certify that the material, which I now submit for assessment onthe programme of study leading to the award of Doctor of Philosophy, is entirely my own work and has not been taken from the work of others save to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Declan Bogan Date: June 9th 1996

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I would like to dedicate this thesis to my parents for all their love and support through it all, for the encouragement, help and understanding. Thank you. They encouraged me to just be happy in whatever I wanted to do whether it involved going for it, giving up, especially just to enjoy life. To Bill and Ted - my source of antibodies, thank you. To Ciaran and Lorraine, thank you. I would not have been able to complete this work with the same enthusiasm as I started it without the regular encouragement and 'shake-up' from Richard. Thank you for your support, dedication to my work and the informal chats. Thank you also for the opportunity and your faith in me.

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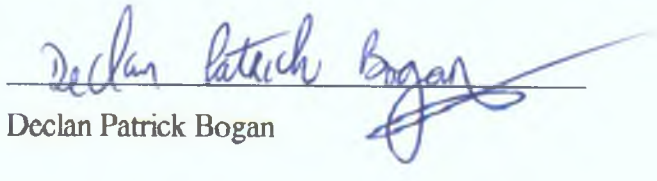
Forever yours,



Declan Patrick Bogan

ABSTRACT

The research presented in this thesis has centered on the development of newer and better techniques for the determination of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide The methods developed were applied to clinical studies of biological fluids including serum, plasma and urine and after *in vitro* metabolism of coumarin and 7-hydroxycoumarin The techniques employed include capillary electrophoresis (CE), high-performance liquid chromatography (HPLC) and antibody-based immunoanalytical techniques

A separation based on capillary electrophoresis (CE) was developed for determining free and total 7-hydroxycoumarin in serum and urine after the *in vivo* metabolism of coumarin The compound was extracted into ether, evaporated to dryness and reconstituted into phosphate buffer before analysis by CE The separation method developed was then applied to the determination of 7-hydroxycoumarin, without sample clean-up, after *in vitro* metabolism of coumarin by liver microsomal suspensions The inter-species and inter-individual variation in coumarin metabolism was assessed A large variation within species (human microsomal preparations - n = 5) and between species (n = 10) was found Another CE method was developed for the direct determination of free 7-hydroxycoumarin and conjugated 7-hydroxycoumarin in urine after *in vivo* metabolism of coumarin This method was also applied to the determination of 7-hydroxycoumarin-glucuronide after *in vitro* metabolism of 7-hydroxycoumarin by uridine diphosphate glucuronyl transferase in a crude enzymatic preparation from liver

High-performance liquid chromatography was used for the determination of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine, plasma and serum. An isocratic separation method was utilised for the determination of total 7-hydroxycoumarin after the administration of 7-hydroxycoumarin to patients diagnosed with a range of cancers The method was used to determine the pharmacokinetic profile of serum levels of the drug A range of 7-hydroxycoumarin doses were given (100 mg - 7000 mg) and the interindividual variations in 7-hydroxycoumarin metabolism were determined Separations were based on enzymatic deconjugation of 7-hydroxycoumarin from the glucuronide form, followed by extraction into ether, evaporation to dryness, and reconstitution into methanol before analysis

by HPLC A gradient separation method was also developed for the direct determination of coumarin, 7-hydroxycoumarin, and 7-hydroxycoumarin-glucuronide in urine, plasma and serum after the *in vivo* metabolism of coumarin and 7-hydroxycoumarin The method was also used for the determination of 7-hydroxycoumarin-glucuronide after the *in vitro* metabolism of 7-hydroxycoumarin by a crude enzymatic preparation from liver

A 7-hydroxycoumarin-thyroglobulin protein-drug conjugate was prepared It was used in the generation of rabbit polyclonal antibodies Antibodies raised against the protein-drug conjugate were screened against another protein-drug conjugate (bovine serum albumin-7-hydroxycoumarin) to determine the antibody titre The antibodies raised were purified from rabbit serum, characterised and utilised in an enzyme-linked immunosorbent assay

ABBREVIATIONS

Ab	antibody
Ag	antigen
Ag/AgCl	silver silver chloride reference electrode
BIAcore	biospecific interaction analysis
BSA	bovine serum albumin
ca	approximately
CE	capillary electrophoresis
CMC	critical micellar concentration
CZE	capillary zone electrophoresis
ELISA	enzyme-linked immunosorbent assay
EOF	electroosmotic flow
Fab	variable portion of IgG molecule
Fc	constant portion of IgG molecule
GC	gas chromatography
GC-MS	gas chromatography with mass spectral detection
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
HRP	horse-radish peroxidase
IgG	immunoglobulin of the G class
IR	infrared analysis
MECC	micellar electrokinetic capillary chromatography
NADPH	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
7-OHC	7-hydroxycoumarin
7-OHCG	7-hydroxycoumarin-glucuronide
7-OHC-BSA	7-hydroxycoumarin-bovine serum albumin protein conjugate
pNPP	paranitrophenol phosphate
% R S D or RSD	percentage relative standard deviation
S D or St Dev	standard deviation
TLC	thin layer chromatography
UV	ultraviolet
UDPGA	uridine diphosphate glucuronic acid
UDPGT	uridine diphospahte glucuronyl transferase

UNITS

µg/ml or ug/ml	micrograms per millilitre
mA	milliamperes
mg	milligrams
kDa	Kilodaltons
Da	Daltons
° C	degrees centigrade
Kg	kilogram
cm	centimetre
µm	micron, micrometre
cm^{-1}	wavenumber per centimetre
µl	microlitre
µamps	microamperes
L	litre
mol	mole
M	molar
µM	micromolar
mM	millimolar
ppm	parts per million
min	minutes
h	hour
A U	absorbance units
RU	response units
nm	nanometres
MHz	megahertz
pH	log hydrogen ion concentration
V	volts
kV	kilovolts
rpm	revolutions per minute
p s i	pounds per square inch
g	times gravity
A/A_0	absorbance divided by the maximal absorbance
v/v, w/v	dilution percentage as volume or weight per volume

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Summary

1.0 INTRODUCTION

The ubiquitous nature of coumarin and its many derivatives, in industry, medicine, and in the environment, have led many investigators to search for methods of analysis of coumarin and its derivatives (Casley-Smith and Casley Smith, 1986, Murray, 1989, Egan *et al* , 1990, Keating and O'Kennedy, 1996) The methods thus far developed cover a wide range of analytical techniques, encompassing more traditional methods, e g paper chromatography, or colourimetric approaches, and currently available general techniques, e g high-performance liquid chromatography, capillary electrophoresis, and immunochemical, and some specific methods e g centrifugal partition chromatography There is a need to determine coumarin and its derivatives in assessing their clinical efficacy, their effects on foodstuffs, and their industrial applications The methods used need to be applicable to a variety of matrices, to a range of concentrations, sensitive, and they need to be reproducible, reliable, and accurate In this chapter structures, nomenclature and occurrence of coumarins are described The principle section deals with the broad range of analytical methods available for coumarin analysis These are critically examined in relation to their application to the analysis of coumarin, and some of its derivatives, with special reference to 7-hydroxycoumarin

1 0 1 The Structure of the Coumarins

The coumarins belong to a group of compounds known as the benzopyrones, all of which consist of a benzene ring joined to a pyrone ring - a six-membered heterocyclic ring containing one oxygen atom and five sp^2-hybridised carbons Both g- and α- pyrones exist, with the prefixes referring to the position at which the oxygen atom is found Figure 1 0 1 1 shows the structure of α-pyrone

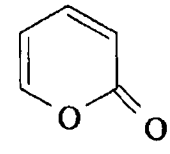


Figure 1 0 1 1 The structure of α-pyrone

Coumarin (Figure 1 0 1 2) and the other members of the coumarin family are benzo-α-pyrones, while the other main members of the group - the flavonoids - contain the g-pyrone group

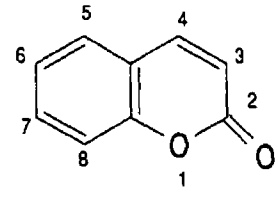


Figure 1 0 1.2 The structure of coumarin

The coumarins display a characteristic fluorescence upon excitation with ultra-violet light, in addition to having a large range of physiological activity Many coumarins are comprised of isoprenoid chains joined to one of the carbons of the nucleus, or to an oxygen atom, for example, a phenolic oxygen The isoprenoid chains may consist of one, two or three units, and many variations of this type of structure are to be found among the coumarins Interactions of the phenyl group with an *ortho*-phenolic group can lead to the formation of another heterocyclic ring Oxidative interaction may also occur As the vast majority of the coumarins are oxygenated at C-7, 7-hydroxycoumarin (Figure 1 0 1 3) is frequently considered to be the parent compound for the more structurally complex members of the family Coumarins may also be found in nature in combination with sugars, as glycosides

The coumarins can be roughly categorised as follows

a) **Simple**

These members of the coumarin family are the hydroxylated, alkoxylated and alkylated derivatives of the parent compound, coumarin, along with their glycosides 7-hydroxycoumarin (Figure 1 0 1 3) is a member of this group

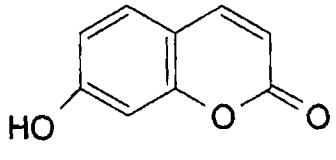


Figure 1 0 1 3 ***The structure of 7-hydroxycoumarin***

b) **Furanocoumarins**

These compounds consist of a five-membered furan ring attached to the coumarin nucleus Most of the members of this group are derivatives of the linear furanocoumarin psoralen (Figure 1 0 1 4), or its more stable angular isomer, angelicin (Figure 1 0 1 5)

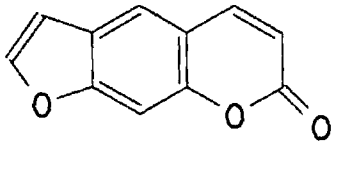


Figure 1 0 1 4 ***The structure of psoralen***

The terms "linear" and "angular" refer to the orientation of the furan ring with respect to the coumarin nucleus The dihydrofuranocoumarins are also members of this group

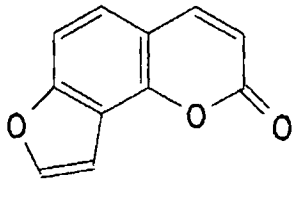


Figure 1 0 1 5 ***The structure of angelicin***

c) **Pyranocoumarins**

Members of this group are analogous to the furanocoumarins, and contain a six-membered ring As with the furanocoumarins, linear and angular types exist (Figure 1 0 1 6 and 1 0 1 7)

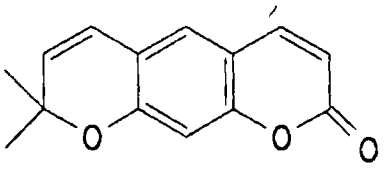


Figure 1 0 1 6 ***The structure of xanthyletin (linear)***

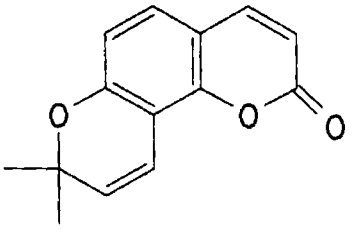


Figure 1 0 1 7 ***The structure of seselin (angular)***

d) **Coumarins substituted in the pyrone ring**

Examples of this group include 4-hydroxycoumarin (Figure 1 0 1 8) and the 3-methyl-4-hydroxycoumarins, such as autumnariol (Figure 1 0 1 9)

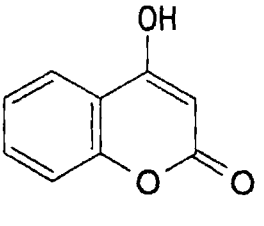


Figure 1 0 1 8 ***The structure of 4-hydroxycoumarin***

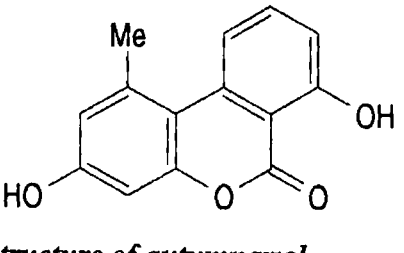


Figure 1 0 1 9 ***The structure of autumnariol***

1 0 2 Nomenclature

Coumarin compounds are known by their common generic titles, generally related to their source, as well as their standard IUPAC names This is illustrated with the nomenclature of coumarin itself The parent compound, coumarin, is variously known as 2-oxo-1,2-benzo-pyran, 5,6-benzo-α-pyrone, 5,6-benzo-2-pyrone, 1,2-benzopyrone, 2H-1-benzopyran-2-one, cis-*o*-coumaric acid lactone, 2H-benzo[*b*]pyran-2-one, coumarinic anhydride and 2H-chromen-2-one and coumarin Members of the coumarin family have been isolated from hundreds of species of plants and microorganisms, giving rise to another problem in the naming of the compounds Many of the coumarins that have been isolated are known by common names, which are generally based upon the latin or colloquial name of the plant in which they have been discovered, or from the place where the plant grows, resulting in the same compound having several different trivial names Frequently, the trivial names which have been assigned to these compounds give little or no information as to its structural composition, and in some cases may even be misleading For example, 7-hydroxycoumarin (Figure 1 0 1 3) is also known as umbelliferone, skimmetin, hydrangin and Dichrin A

1 0 3 Occurrence

Although some have been discovered in animals and microorganisms, most of the naturally occurring coumarins have been extracted from higher plants The richest sources have been the Rutaceae and Umbelliferae, where all parts of the plant have been discovered to contain coumarins The coumarins belong to a group known as the secondary products of plant metabolism, the exact roles of which in plant physiology are not clearly understood While being distributed throughout all parts of the plant, the coumarins occur at the highest levels in the fruits, followed - in order of decreasing occurrence - by the roots, stems and leaves In addition, seasonal changes and environmental conditions may affect the occurrence in various parts of the plant

Of the lower plants, neither algae or mosses have been reported to contain coumarins Complex coumarins such as novobiocin and chartreusin have, however, been discovered in

bacteria Several coumarins - most notably, perhaps, the aflatoxins - have been extracted from fungi

The *Castoreum pigments* have been isolated from the yellow secretions of beaver sweat glands Murray *et al* (1988) and Keating and O'Kennedy (1996) gave a comprehensive overview of the chemistry and occurrence of natural coumarins, including a table of the botanical sources of all of the coumarin compounds known at time of publication

1 1 THE ANALYSIS OF COUMARINS - AN HISTORICAL BACKGROUND

The presence of coumarin in plant extracts or as a flavouring component was of interest to the early coumarin analysts Paper chromatography, thin layer chromatography, colourimetric assays and polarography were all used These more traditional analytical methods were applied to the analysis of chocolate (Gardner, 1954), sweet clover (Ferencz and Veres, 1953), flavourings (Nivoli, 1950, Ensminger, 1952, Stoll and Bouteville, 1954, ted Heide and Lemmens, 1954) and plant extracts (Patzak and Neugebauer, 1952, Riedl and Neugebauer, 1952, Svedsen, 1952, Foffani, 1953) The majority of these techniques were applied primarily for the qualitative determination of coumarin and/or its derivatives

The concentration of coumarin and derivatives was determined by colourimetric assays (Ensminger, 1952) or by using the ultraviolet (UV) absorption spectra of the derivative of interest after chromatographic separation (Nivoli, 1950) Coumarin was treated with p-aminophenol to selectively determine it in the presence of vanillin (Nivoli, 1950)

Paper chromatography was also used for the separation of coumarins Separation with butanol acetone water (Swain, 1953), acetic acid butylene glycol water (Berlingozzi and Fabbrini, 1954), or ammonia butanol water (ted Heide and Lemmens, 1954), were some examples of mobile phases used for the separation of coumarin and its derivatives in organic flavourings or in plants After separation of coumarin from other endogenous species present in the matrix of interest it is possible to visualise coumarin after treatment with an alkali or after photocatalysis Coumarin can be visualised by treating the chromatogram, after separation, with alkali e g KOH, or Na_2CO_3 (Mitchell, 1953, Berlingozzi and Fabbrini, 1954, ted Heide and Lemmens, 1954, Stoll and Bouteville, 1954) The coumarinic salt produced is fluorescent and the intensity can be used to determine coumarin concentrations Photocatalysis was another technique (Feigl *et al* , 1955) utilised for coumarin and 7-hydroxycoumarin detection after paper chromatographic separation Here the chromatogram was treated with an alkali, irradiated to form a trans isomer that

fluoresces blue for 7-hydroxycoumarin and yellow-green for coumarin under UV light The limit of detection of the method was 5 ng

Derivatisation of coumarin was also investigated for use in qualitative and quantitative analysis (Glenn and Peake, 1955, Wild, 1964, Anger and Richter, 1965) The analysis was based on the formation of acidic products, amides of ethylenediamine, or coloured products After coumarin was dissolved in ethylenediamine, a rapid visual indicator, or the potentiometric response of the derivative, was utilised for its determination (Glenn and Peake, 1955) The coloured diazocoupled product (Wild, 1964), formed between diazotised sulfanilic acid and coumarin (Wild, 1964), had a linear detection range between 0 1 - 0 7 g/l. The sodium salt of coumarinic acid (formed by reaction of coumarin and alkali) was reacted with 2,6-dichloroquinone-4-chloroimide to form a blue indophenol dye (Anger and Richter, 1965) Their method had a linear detection range between 2-10 mg/ml

The emergence of more sophisticated analytical technology in the nineteen sixties and seventies led to its application for the determination of coumarin and its derivatives Gas chromatography was applied to the determination of coumarin as a contaminant of flavourings (Bucci *et al* , 1965, Johansen, 1965) Limits of detection were in the order of 0 2 mg, or 0 01% of sample with an electron capture detector

Today high performance liquid chromatography, gas chromatography, biosensors, and, capillary electrophoresis are routinely used for the analysis of coumarin and its analogues Method development, data handling and the applicability of these techniques are made more straightforward by the development of computer controlled systems

1 2 CHROMATOGRAPHIC ANALYSIS OF COUMARINS

One of the most widespread analytical tools is chromatography The basis of chromatography is the separation of components in a mixture and their selective detection Table 1 2 1 is a table summarising some of the chromatographic techniques available that have been used for the determination of coumarin and 7-hydroxycoumarin

1.2.1 Thin-Layer Chromatography

The principle of thin-layer chromatography (TLC) is the separation of compounds on a coated solid surface with a liquid carrier phase, typically a silica coated stationary phase, with an organic mobile phase The compound is applied to the solid surface and interaction with the carrier phase, in which the components migrate along the solid surface, enables separation A range of solid and mobile phases have been assessed for their applicability to the separation of coumarin and its derivatives Typically, TLC was used as a purely qualitative method but it is possible to use TLC in quantitative analysis

1.2 1 1 Metabolism of umbelliferone and herniarin

The metabolism of 7-hydroxycoumarin (umbelliferone) and 7-methoxycoumarin (herniarin) was followed by TLC (Indahl and Scheline, 1971) Separation of the principle metabolites was achieved with benzene-glacial acetic acid-water (6 7 3, v/v/v), and with, potassium chloride-glacial acetic acid (100 1 v/v), on thin layer cellulose plates (Sigmacell type 19, and Macherey, Nagel & Co MN 300 plates) After separation, the spots on the TLC plates were observed under UV light (fluorescence at 254 nm) or by spraying with fast blue B salt The different compounds gave a variety of different colours e g 7-hydroxycoumarin (purplish-white), 7-methoxycoumarin (pinkish-white), 6,7-dihydroxycoumarin (greenish-yellow), and 3,4-dihydro-7-hydroxycoumarin (reddish-violet) The method was used for studying the metabolism of 7-hydroxycoumarin in rats after oral and intraperitoneal administration

Method & Application	Mode of Detection	Limit of Detection	Reference
Thin layer chromatography			
In vivo 7-OHC	Colourimetric	1 ng/ml	Johansen *et al* (1965)
metabolism	Fluorescence	5 ng	Cholerton *et al* (1992)
Vanilla flavouring	Fluorescence	500 ng	Indahl *et al* (1971)
	UV absorbance	(Coumarin)	Sherma *et al* (1987)
			Poole *et al* (1993)
High performance liquid chromatography			
In vivo coumarin metabolism	UV absorbance	500 ng/ml (7-OHC)	Moran *et al* (1987), Egan *et al* (1992)
		0 3 ng/ml (7-OHC & 7-OHCG)	Sharifi *et al* (1993)
In vitro metabolism	UV absorbance		Fentem *et al* (1991)
Coumarin	Radiochemical		Lake *et al* (1992), van Iersel *et al* (1994), Steensma *et al* (1994)
Coumarin in Foodstuffs	UV absorbance	0 6 ng/ml	Thompson *et al* (1988) Mazza (1984),
Plant extracts		1 ng/ml	Gamache *et al* (1993) Archer (1988),Nykolov *et al* (1993)
Gas chromatography			
Coumarin in Flavours/Fragrances	Mass Spec FTIR	7 7 ng/ml	Hawthorne *et al* (1988), Grundschober (1991)
Tobacco	FID/ Mass Spec	0 05 mg/g	Christakopoulus *et al* (1992)

Table 1 2 1 Summary of the chromatographic methods of analysis of coumarins Table of method of separation, detection, the limits of detection (where available) and the reference

1.2 1 2 TLC of vanilla flavourings

High performance silica gel TLC was used to qualitatively and quantitatively determine coumarin in real and artificial vanilla flavourings (Sherma *et al*, 1987) Samples and standards were spotted onto Whatman LHPKD preadsorbent silica gel plates Separation was carried out with toluene-methanol (97 3, v/v) and visualised under UV light by spraying with NaOH Coumarin was detected as a fluorescent or coloured zone and

quantified by scanning with a densitometer The limit of detection was 5 ng for fluorescence and 500 ng for absorbance Belay *et al* (1993) determined vanillin and the adulterant, coumarin, in a variety of natural vanilla beans, by TLC They employed an automated multiple development (AMD) system which optimises the separation selectivity throughout the chromatogram for mixtures of a wide polarity range The whole process is automated which ensures a high degree of reproducibility Separation was carried out with a stepwise mobile phase change between chloroform, ethyl acetate, 1-propanol, acetic acid and hexane They were (Belay *et al*, 1993) able to separate and identify coumarin from other vanilla extracts e g vanillin, ethyl vanillin, vanillyl alcohol, vanillic acid, 4-hydroxybenzyl alcohol 3,4-dihydroxybenzaldehyde, 4-hydroxybenzoic acid, and piperonal.

A chloroform, ethyl acetate, propan-1-ol (96 2 2, v/v/v) mobile phase was also used for the separation of vanilla extracts (Poole *et al*, 1993) by TLC The method allowed the determination of the authenticity of natural vanilla extracts as opposed to synthetic flavour compounds which can only be found in counterfeit vanilla flavourings (coumarin is considered as an adulterant in counterfeit vanilla flavouring) Whatman HP-K high performance silica gel plates were used for the separation of coumarin from the other species present in the flavourings Plates were analysed at 280 nm or as 2,4-dinitrophenylhydrazone derivatives at 500 nm with a scanning densitometer

1 2 1 3 TLC analysis of 7-hydroxycoumarin in urine

TLC was also used for the analysis of 7-hydroxycoumarin after *in vivo* metabolism of coumarin (Cholerton *et al*, 1992) 64 patients were treated with 2 mg of coumarin Samples were treated with β-glucuronidase, and extracted into chloroform. Separation was carried out on silica plates (precoated high-performance TLC glass-backed silica gel 60 F_{254}) with a mobile phase of chloroform-water-ethyl acetate-acetic acid (24 12 6 0 5 v/v) Quantitative results were obtained by fluorescence densitometry The chromatographic lanes were scanned at a wavelength of 313 nm using a mercury lamp in the reflection mode in the presence of a K400 secondary filter Peak heights were compared to those of 7-hydroxycoumarin standards The limit of detection was 1 ng/ml with a linear range of 5 ng/ml - 4000 ng/ml.

1 2 2. High Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a technique whereby molecules can be separated by exploiting their physical and / or chemical differences (Knox, 1980, Willard *et al*, 1988, and Meyer, 1988) For a review on the different modes of HPLC available see section 4 0 HPLC has been applied to the analysis of coumarin in flavourings, in plant extracts and in *in vivo*, and *in vitro* metabolism studies Separation was achieved on a variety of stationary phases e g C_{18}, C_8 with many different mobile phases e g based on methanol, or acetonitrile Detection systems utilised included, UV detection, radiochemical detection, mass spectral detection, fluorescence detection, and, electrochemical detection An excellent review on the determination of many coumarin derivatives by HPLC between 1983 and 1990 are covered by Shkarenda *et al* (1993) The conditions for the separation of complex mixtures of coumarins and the use of coumarins in the HPLC analysis of natural and synthetic compounds are discussed The coumarin derivatives analysed include furanocoumarins, hydroxycoumarins, pyranocoumarins, and isocoumarins Detection wavelengths, column selection, mobile phases, sources and the presence of an internal standard are all included

1.2.2 1 Metabolism studies

Hepatic microsomes play a major role in role in the metabolism of coumarin to a variety of metabolites (see Figure 3 2 1 1) The metabolic pathway is dependent on the species (Cohen, 1979, Kaipainen *et al*, 1985, Egan *et al*, 1990, Pelkonen *et al*, 1996) HPLC has been adapted to determine the many metabolites both for studies on the *in vivo* and *in vitro* metabolism of coumarin This is achieved by determining coumarin metabolites after *in vivo* or *in vitro* metabolism (Figure 1 2 2 1)

1 2 2 1 1 Metabolism in vivo

Moran *et al* (1987) and Egan and O'Kennedy (1992) both described methods for the determination of 7-hydroxycoumarin in urine after the *in vivo* metabolism of coumarin in man Initial sample preparation steps and long incubation steps made the method of Moran

et al (1987) very time consuming Large samples of urine were required, and large amounts of β-glucuronidase were also needed to liberate the glucuronide conjugate from 7-hydroxycoumarin The method of Egan and O'Kennedy (1992) made major improvements on this method It shortened the total time of preparation by at least 16 hours The amount of β-glucuronidase was reduced from 20,000 Units/ml to 5000 Units/ml and the incubation time was reduced from 16 hours for the method of Moran *et al* (1987) to 0 5 hours for the method of Egan and O'Kennedy (1992)

Direct extraction into diethyl ether (Egan and O'Kennedy, 1992), rather then freeze drying (Moran *et al*, 1987), was the preferred method of sample preparation before the application of the sample onto the HPLC Samples were separated with mobile phases based on a methanol water acetic acid mix [300 700 2 (v/v/v), Egan and O'Kennedy, (1992), and 200 300 1 (v/v/v) for Moran *et al* (1987)] The limit of detection of the method of Egan and O'Kennedy (1992) was 500 ng/ml with a linear detection range between 0 - 100 mg/ml Sharifi *et al* (1993) developed a method that avoided the deconjugation step entirely for the determination of coumarin and some of its metabolites (7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide, 3-hydroxycoumarin) in urine

Extraction of the glucuronide conjugate from rabbit urine, giving a pure standard to work with, allowed the study of samples containing 7-hydroxycoumarin-glucuronide without having to deconjugate The urine was prepared by altering its pH to 4 5 and then diluting the sample with water and methanol Separation was achieved with a methanol water tetrahydrofuran acetic acid (45 40 10 5 v/v/v/v) mobile phase Sharifi *et al* (1993) used a C_8 reverse phase column to separate the individual components of interest whereas Moran *et al* (1987) and Egan and O'Kennedy (1992) both used C_{18} reverse phase columns

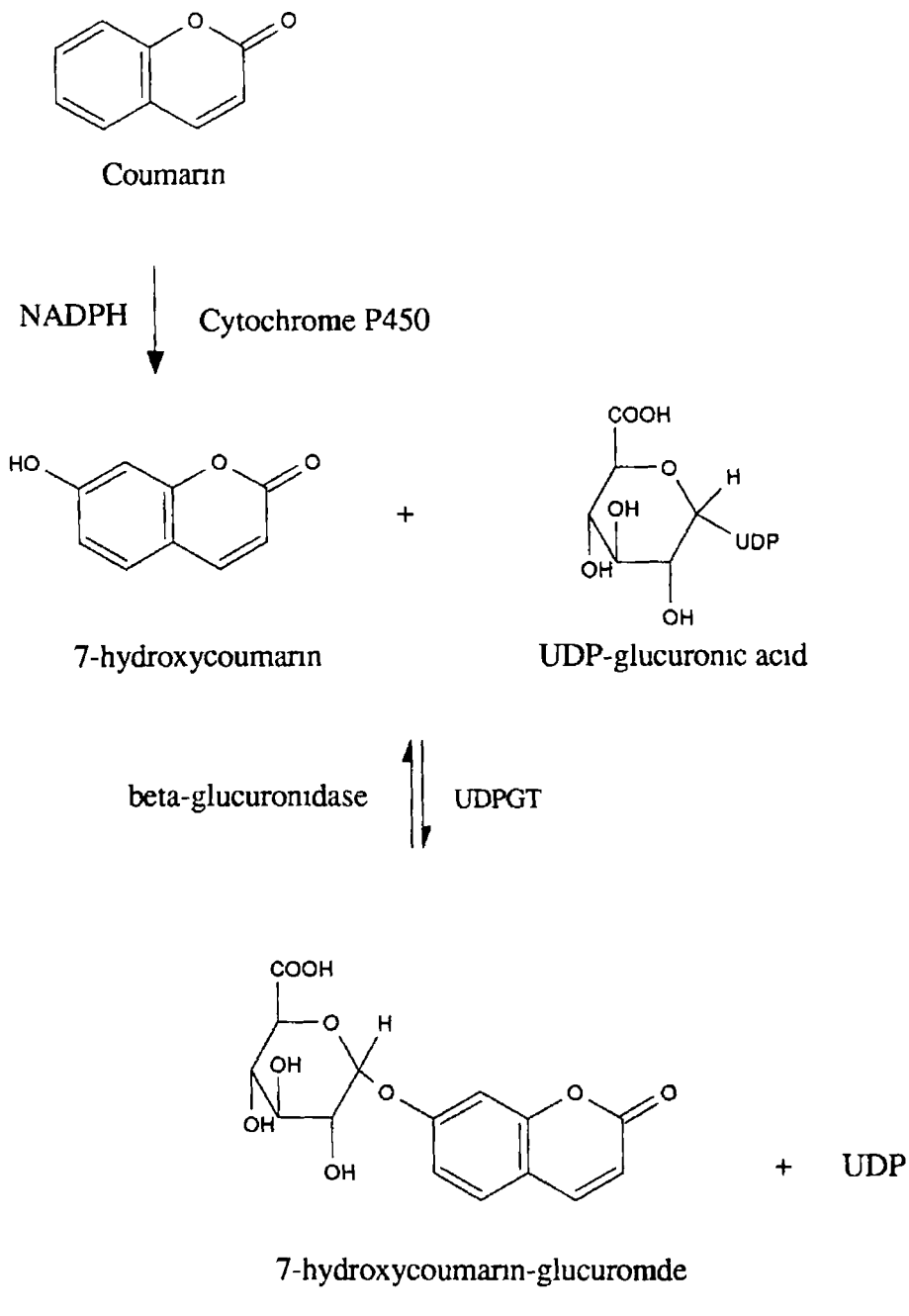


Figure 1 2 2 1 ***The reaction sheme for an in vitro or in vivo pathway of coumarin metabolism to 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide respectively The Figure shows both the Phase I metabolism (see section 3 2 1) and the Phase II metabolism of coumarin (see section 3 2 3), the metabolism of 7-hydroxycoumarin to 7-hydroxycoumarin-glucuronide, and the species necessary for the metabolism of both coumarin and 7-hydroxycoumarin to their respective metabolites The pathway illustrated is the principle metabolic fate of coumarin in man***

The limits of detection for the method of Sharifi *et al* (1993) were far superior to those of Egan and O'Kennedy (1992) or Moran *et al* (1987) They were able to detect down to 0 3 ng/ml with a linear range between 0 6 and 26 ng/ml (250 - 10000 nmol/ml) for 7-hydroxycoumarin-glucuronide, and similarly for coumarin and 7-hydroxycoumarin Lamiable *et al* (1993) utilised a different mobile phase (acetonitrile phosphate buffer), extraction into hexane, and reconstitution into hydrochloric acid for the determination of coumarin in plasma (limit of detection of 0 3 ng/ml) They used a C_8 column as compared with the methods of Egan and O'Kennedy (1992) and Moran *et al* (1993) who used C_{18} columns All the above methods used UV detection at 280 nm or 320 nm.

Hiller and Cole (1995) described a method for the determination of 7-ethoxycoumarin and its metabolites after phase I and phase II metabolism. Their method utilised short-column gradient elution HPLC for the separation of 7-ethoxycoumarin, 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and 7-hydroxycoumarin-sulphate in liver-slice incubates Separation was carried out on a 33 mm X 4 6 mm -i.d , 3 µm particle size, LC-8 HPLC column The mobile phases used for the gradient elution consisted of acetonitrile, tetrabutyl ammonium phosphate and acetic acid The different compounds were prepared in Krebs-Henseleit buffer containing human or mouse liver-slices and analysed directly by HPLC without any sample clean-up

1 2.2 1 2 Metabolism in vitro

High-performance liquid chromatography has also been used to follow the *in vitro* metabolism of coumarin by hepatic microsomes Fentem *et al* (1992) showed the complete separation of a standard mixture of the majority of coumarin metabolites Separation was carried out on a 250 mm X 4 6 mm Spherisorb 5 ODS-2 column using a linear gradient elution of formic acid water methanol, and detection at 280 nm. O-hydroxyphenylacetaldehyde was found to be a major metabolite of coumarin formed by rat, human, and gerbil liver microsomes (Fentem *et al*, 1991) This was determined by separation as above (Fentem *et al*, 1992) but with mass spectral analysis of the metabolite isolated from the rat liver microsomal incubation HPLC has been used for the

determination of cytochrome P-4502A6 activity, as well as for the determination of other coumarin metabolites

Metabolism of radiochemical labelled [3-^{14}C]coumarin by hepatic microsomes from various species was also studied by HPLC (Lake *et al*, 1992, van Iersel *et al*, 1994, Steensma *et al*, 1994) The metabolites were determined by comparison of retention times with known standards The concentration of metabolite was quantified by measurement of the radioactivity present in the appropriate HPLC fractions At specific timed intervals the metabolic reaction was terminated by the addition of ice-cold methanol, followed by centrifugation It was possible to monitor the majority of metabolites produced over time to give a pharmacokinetic profile of coumarin metabolism *in vitro*

Evans and Relling (1992) described 7-ethoxycoumarin O-deethylase activity by analysing 7-hydroxycoumarin using fluorescence detection (excitation at 360 nm and an emission at 470 nm) Separation was carried out on a μBondapak phenyl column (300 mm x 3 9 mm I D) Separation was achieved with an isocratic mobile phase of (55 45, v/v) methanol and 0 05 M acetate buffer (pH 4 7) The limit of detection was 70 ng/ml. 7-hydroxycoumarin was extracted into chloroform and this was evaporated to dryness followed by reconstitution into methanol-water (70 30, v/v) before HPLC separation and analysis

1.2.2.2 Coumarin in foodstuffs

Coumarin is the most common adulterant of vanilla extract (Thompson and Hoffmann, 1988) The method of Thompson and Hoffmann (1988) utilised a methanol water acetic acid mobile phase (40/60/0 1 v/v/v), on a C_{18} column, with UV detection and a limit of detection of 0 6 ng/ml (from 3 X signal-to-noise ratio) It was possible to separate coumarin from vanillin as well as other endogenous species in bean extracts Thus, it was possible to use HPLC in the assessment of quality Phenolics and flavanoids are common substituents of plant-derived beverages (Gamache *et al*, 1993), playing a role in the stability, colouration, nutritional value, etc Many coumarin derivatives, including 7-hydroxycoumarin, were separated by HPLC with coulometric array detection Limits of detection were in the order of 1 ng/ml. Electrochemical detection provides selectivity and

sensitivity to the study of trace amounts of these compounds in such matrices Mazza (1984) also describes the separation and determination of coumarin in alcoholic beverages Separation was carried out on a LiChrosorb RP-18 column with a 1 1 water methanol mobile phase

1 2 2 3 Plant extract analysis

Many of the reported HPLC analyses of coumarin, and its derivatives, involved some form of sample preparation The method might require organic solvent extraction (Moran *et al*, 1987, Lander *et al*, 1990, Egan and O'Kennedy, 1992, Nykolov *et al*, 1993, Sharifi *et al*, 1993), precipitation of any protein present (Fentem *et al*, 1991), or filtration (Archer, 1988)

Nykolov *et al* (1993) determined numerous hydroxycoumarins present in the bark of *Fraxinus ornus*, which is a major source of esculin, an anti-inflammatory and vitamin-P-like agent Climatic conditions, and the stage of plant growth will lead to great variations in hydroxycoumarin content The samples were extracted into ethanol, and analysed on a C_8 column, with gradient elution of the components (mobile phases were water phosphoric acid, pH 3 0, and methanol) and UV detection at 210 nm Preparative HPLC was used to purify the individual components Fractions were taken and analysed separately by thin layer chromatography, and by mass spectrometry to characterise each of the hydroxycoumarins

The determination of coumarin in cinnamon and cassia (Archer, 1988) was achieved by separation on a LiChrosorb RP-8 reversed-phase column with a water, methanol, acetonitrile, tetrahydrofuran (60 12 20 8 v/v/v/v) mobile phase Vanillin was used as an internal standard

1.2 3 Gas Chromatography

Gas chromatography (GC) is a technique that allows the separation of thermally stable and volatile organic and inorganic compounds (Willard *et al*, 1988) The early work on coumarin analysis (Johansen, 1965) centred on the separation of coumarin as an adulterant

in flavourings More recent work has focused on coumarin as a flavouring (Hawthorne *et al*, 1988, Grundschober, 1991), as a fragrance (Hawthorne *et al*, 1988, Jirovetz *et al*, 1992), in tobacco and in vegetables (Christakopoulus *et al*, 1992) Separations were typically carried out on fused-silica capillaries with helium as the carrier gas The temperature gradients for the different methods varied from 8 °C/min or 10 °C/min up to a final temperature of 300 °C (Christakopoulus *et al*, 1992, Weinberg *et al*, 1993)

m/z	**Range of relative abundance**
147	7 - 12
146	66 - 100
119	8 - 10
118	82 - 100
90	28 - 41
64	30 - 40
63	8 - 12
62	5 - 15
59	8 - 15
44	7 - 11
43	5 - 14
39	11 - 16

Table 1 2 3 1 Mass spectral analysis data for relative abundances of coumarin ions The results are given as the mass/charge ratio (m/z) and the relative abundances of each ion

1 2 3 1 Flavours and fragrances

Supercritical fluid extraction (SFE) was used as the preparation step to determine coumarin in some natural flavour and fragrance compounds (Hawthorne *et al*, 1988) The samples were extracted into 300 atmospheres of supercritical carbon dioxide at 45 °C The extraction chamber outlet restrictor capillary was connected to the standard on-column injection port of the gas chromatogram allowing for an on-line purification and separation This reduces sample loss due to sample transfer after extraction The sample is then separated and detected by either mass spectrometry or flame ionisation (FID) The method was used for the determination of coumarin in cinnamon and in rosemary and for the analysis of spices, chewing gum, spruce needles, and cedar wood Separation

was carried out on the GC with a gradient temperature program from 70 °C to 320 °C at 8 °C/min 1 14 ± 12% mg of coumarin was detected per g of rosemary

The efficacy of using GC was also assessed as a tool for the accurate identification of coumarin, and other flavourings (Grundschober, 1991) In the report by Grundschober (1991) coumarin is separated by GC with mass spectral (MS) and Fourier transform infra-red (FTIR) detection to distinguish coumarin from other flavourings present in the species of interest Infra-red bands and their intensity and mass spectral coumarin ion relative abundances are included The relative abundances for coumarin ion profile after mass spectral analysis are given in Table 1 2 3 1 and the infra red bands observed for coumarin are given in Table 1 2 3 2

The effective blood concentration of coumarin in mice, after the inhalation of coumarin, is described by Jirovetz *et al* (1992) Sandalwood oil was added into the cages of mice, and blood samples were taken and assessed for coumarin content The levels of coumarin found in the blood were 7 7 ng/ml (± 2 ng/ml), after the addition of coumarin (3 0 g) into the mouse cage Samples were separated and detected by a variety of methods, including FTIR, MS and atom emission detection

Infra-red bands (cm^{-1})
825 (w)
877 (w)
919 (w)
1096 (m)
1173 (w)
1265 (w)
1451 (w)
1608 (w)
1778 (w)
3079 (w)

Table 1 2 3 2 A list of the infra-red bands (cm^{-1}) observed for coumarin as analysed by Fourier transform infra-red analysis, (w = weak band, m = medium band)

1 2 3 2 Tobacco and vegetable juice analysis

Thin-layer chromatography and high-performance liquid chromatography were used to purify coumarin from tobacco samples before their analysis by gas chromatography (Christakopoulus *et al*, 1992) A chloroform-hexane (25 75, v/v) solution was used to extract the coumarin from the various tobacco leaf samples chosen The HPLC mobile phase was based on a chloroform-hexane gradient [6 94, (v/v) at T_0, 40 60 at T_{18}, and at T_{24} 6 94] Coumarin was separated on a silica gel TLC plate, with a pentane-ethyl acetate (70 30, v/v) mobile phase The coumarin fraction from the HPLC and the coumarin TLC spot were then analysed by GC-MS The limit of detection of the method was 0 05 mg/g tobacco The identity and purity of coumarin were confirmed by nuclear magnetic resonance (NMR), and mass spectrometry (MS) Radiolabelled ^{13}C-coumarin was used as an internal standard for mass spectral determination of coumarin Weinberg *et al* (1993) describe the analysis of coumarins in vegetable juices The coumarins present in vegetable juices includes umbelliferone, psoralen, and bergapten The coumarins were extracted into methylene chloride, and analysed by GC-MS

1.2 4 Capillary Electrophoresis Analysis

There has only been a limited amount of work reported on the analysis of coumarin and its derivatives by capillary electrophoresis This powerful analytical technique is a relatively new addition to the analyst and it is gaining in widescale acceptance for the determination of many species in a multitude of areas (Li, 1992, Landers, 1993, Weinberger, 1993) Several researchers have applied this tool to the separation of coumarin, 7-hydroxycoumarin and warfarin in a variety of matrices For a review on the different modes of CE separation see section 3 0

1.2 4 1 Separation of coumarins in plant extracts

Seven coumarins were separated by micellar electrokinetic capillary chromatography [MECC] (Morin and Dreux, 1993) Coumarin, and several of its hydroxy- and methyl-derivatives were separated by a phosphate-borate (pH 7 0) and sodium dodecyl sulphate (SDS, anionic surfactant) as electrolyte The eluent was monitored at 195 nm. The samples were analysed on a 70 cm untreated capillary with an applied voltage of 18 kV

These neutral coumarins are separated based upon the differential partitioning between the SDS micelle hydrophobic interior and the aqueous mobile phase

1 2 4 1 Using 7-hydroxycoumarin to determine separation efficiencies

The electroosmotic velocity occurring in capillaries made from polyfluorocarbon, polyethylene or polyvinylchloride was determined by the use of electrically neutral marker substances in dependence on the pH in aqueous solutions (Schutzner and Kenndler, 1992) The fluorescent characteristic of 7-hydroxycoumarin was exploited 7-hydroxycoumarin is determined as it is neutral in the acidic pH range up to 5 8 The pH and composition of electrolyte solutions are changed and the effective influence of the coating at the capillary surface was assessed

1.2 4 3 Warfarin determination in plasma

3-(-α-acetonylbenzyl)-4-hydroxycoumarin (warfarin) is widely used as an anticoagulant As a racemic compound it was necessary to develop the separation of the two forms of warfarin based on chiral capillary electrophoresis (Gariel *et al*, 1993) A methylated b-cyclodextrin-containing electrolyte was used to successfully resolve the two isomers Plasma samples were analysed on a 72 cm fused silica capillaries The limit of detection was 0 65 mM for each enantiomer The optimisation of the separation is described Varying of voltage, electrolyte ionic strength and content has a dramatic effect on separation

1 3 SPECTROSCOPY

Absorbance, fluorescence, and luminescence of any compound is dependent on the interaction of electromagnetic waves and matter (Banwell, 1983) Interactions are dependent on the molecular structure of the compound and unique for all compounds Coumarin and all its derivatives have their own unique spectroscopic properties that can be exploited for their determination Table 1 3 1 summarises the spectroscopic methods available for the determination of 7-hydroxycoumarin or derivatised coumarin

Method & Application	Mode of Detection	Reference
Spectrofluorimetry		
Pharmacokinetics of coumarin metabolism	Fluorescence of 7-OHC & derivatised coumarin	Tan *et al* (1976), Ritschell *et al* , (1977, 1981), Kaipainen *et al* (1985)
Glucuronidation of 7-OHC	Fluorescence of 7-OHC	Conway *et al* (1984)
In vivo metabolism of coumarin	Fluorescence of 7-OHC	Rautio *et al* (1992), Merkel *et al* (1993, 1994), Iscan *et al* (1994)
	Microplate-assay	Egan and O'Kennedy (1993a)
	TLC separation	Cholerton *et al* (1993)

Table 1 3 1 Summary of the spectroscopic methods of analysis of coumarins Table of the methods of detection, the application and the references

1 3.1 Spectrofluorimetry

1 3 1 1 Pharmacokinetic and biopharmaceutical studies

Tan *et al* (1976) described a method for the quantitative analysis of coumarin and 7-hydroxycoumarin, and mixtures thereof, in whole blood Coumarin and 7-hydroxycoumarin were selectively isolated from blood by solvent extraction and analysis was based on the measurement of fluorescence of the coumarin fluorophore or 7-hydroxycoumarin (which is fluorescent) The coumarin is extracted into ether and

evaporated to almost dryness with a constant nitrogen flow at low heat 1 ml of methanol is added and the evaporation of the ether is completed 5 0 ml of 2 N methanolic KOH is then added to the solution Coumarin under alkali conditions is then exposed to UV light The fluorescence is determined at activation and emission wavelengths of 361 nm and 491 nm, respectively The linear range for coumarin determination is 0 02 µg/ml - 0 2 µg/ml 7-hydroxycoumarin is extracted into ether also However, a second extraction step is then used to isolate 7-hydroxycoumarin before fluorescence detection Glycine buffer (pH 10 0) is added to the ether layer and centrifuged The glycine layer is removed and fluorescence is determined at activation and emission wavelengths of 370 nm and 450 nm, respectively The linear range for 7-hydroxycoumarin was 1 ng/ml - 10 ng/ml. For the analysis of a mixture of coumarin and 7-hydroxycoumarin the procedure is as just described with the ether layer used for coumarin determination and 7-hydroxycoumarin determined by its the fluorescence in glycine buffer The method was shown to be sensitive, and reproducible with little interference from other constituents in blood and there was no need to precipiate out the proteins in the blood Other umbelliferone conjugates or other hydroxycoumarins do not interfere

The spectrofluorimetric method of Tan *et al* (1976) was used to determine the pharmacokinetics of coumarin after peroral and intravenous administration (Ritschel *et al*, 1977) in man Levels of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide were determined by spectrofluorimetry Samples were analysed after extraction for free 7-hydroxycoumarin and after treatment with β-glucuronidase to liberate 7-hydroxycoumarin from the glucuronide conjugate for total 7-hydroxycoumarin content determination

Coumarin must be derivatised and irradiated with UV light before it can be analysed by fluorimetry (Ritschel *et al*, 1981) Coumarin was firstly extracted into anhydrous ether, then evaporated to dryness, and finally reconstituted into methanol. An alkali solution of 2N KOH was added to the reconstituted sample and irradiated with UV light After irradiation, it was analysed by fluorimetry with an excitation wavelength at 361 nm and emission at 491 nm. 7-hydroxycoumarin can be analysed after clean-up without the need for derivatisation The excitation and emission wavelengths for 7-hydroxycoumarin are

370 nm and 450 nm respectively This procedure was utilised for determining binding of coumarin and 7-hydroxycoumarin to proteins (in human serum) and to erythrocytes and for assessing their solubility in water, phosphate buffer, and human serum.

Coumarin 7-hydroxylation activity in the liver microsomes of 8 different species was investigated by Kaipainen *et al* (1985) An incubation solution containing coumarin, NADPH, $MgCl_2$, and the microsomal preparation was prepared At predefined times the reaction was stopped by precipitating the solution with trichloroacetic acid NaOH-glycine buffer, (pH 10 3), was added to the solution and fluorescence was determined at excitation and emission wavelengths of 390 nm and 440 nm, respectively Antibodies were raised, in mice, against cytochrome P450, with high activity against coumarin 7-hydroxylation The microsomes were preincubated with the antibody Inhibition of coumarin 7-hydroxylase activity was assessed in the microsomes from the different species There was almost 100 % inhibition of coumarin 7-hydroxylation in some species and little if any in the rat or rabbit microsomes

1 3 1 2 Glucuronidation studies

The major site of glucuronidation (a detoxification pathway that makes compounds more hydrophilic and more readily excretable in urine) is the liver (see section 3 2 3) The majority of coumarin administered in man is excreted as 7-hydroxycoumarin-glucuronide (Egan and O'Kennedy, 1992) A novel method was developed that allowed the monitoring of the decrease in 7-hydroxycoumarin content (Conway *et al* , 1984) A fluorimeter is connected via optical fibres to micro-light guides that were placed on the surface of liver tissue samples It was possible to monitor the fluorescence of 7-hydroxycoumarin As the 7-hydroxycoumarin was converted to 7-hydroxycoumarin-glucuronide the decrease in fluorescence could be observed (Conway *et al* , 1984) One strand of the micro-light guide is connected to a 100-W mercury arc lamp and the other strand to a photomultiplier Excitation and emission wavelengths were at 366 nm and 450 nm, respectively Different parameters were changed to assess the differences in glucuronidation in the different liver regions Glucuronosyltransferase activity was determined by measuring 7-hydroxycoumarin-glucuronide spectrofluorimetrically after treating it with β-glucuronidase

in a fluorimeter Maximal rates of glucuronidation in periportal and pericentral regions of the liver lobule were 9 6 and 35 μmoles/g/hr, respectively

1 3 1 3 Urine analysis

Several investigators have used the fluorescence characteristics of 7-hydroxycoumarin for its determination in urine A spectrofluorimetric assay was used to assess interindividual variability in coumarin 7-hydroxylation by Rautio *et al* (1992) and Iscan *et al* (1994) Samples of urine were treated with β-glucuronidase before extraction into chloroform and analysis Results were compared to analysis of the same samples by a HPLC method (Rautio *et al*, 1992) Samples were analysed on a Interchrom Nucleosil C_{18} column with UV detection at 313 nm. Samples were separated by gradient elution [buffer A - 20 % of a 1 5 % acetic acid (pH 4 85, adjusted with NH_4OH) and 80 % buffer B buffer B - 50 % buffer A + 50 % acetonitrile] Different doses of coumarin were used in a preliminary experiment to study the relationship between the dose and the 7-hydroxycoumarin excretion (Rautio *et al*, 1992) However, both groups administered a single 5 mg dose of coumarin for the interindividual variability study

In the two studies 100 (Rautio *et al*, 1992) and 110 people (Iscan *et al*, 1994), respectively, were administered coumarin and the production of total 7-hydroxycoumarin was determined There was a large interindividual variability in the metabolism of coumarin to 7-hydroxycoumarin Up to 80 % of the 7-hydroxycoumarin formed was excreted in the first two hours 59 8 % (± 21 5 %) of the administered coumarin was derivatised to 7-hydroxycoumarin or 7-hydroxycoumarin-glucuronide (Iscan *et al*, 1994) Coumarin 7-hydroxylation was found to be lower in males and in cigarette smokers (Iscan *et al*, 1994), however, Rautio *et al* found no statistical difference between male and female coumarin 7-hydroxylase activity

Merkel *et al* (1993, 1994) also investigated 7-hydroxycoumarin production by analysing urinary excretion of the coumarin metabolite Samples were prepared by a similar method to that of Rautio *et al* (1992) Merkel *et al* (1994) report that grapefruit juice flavanoids inhibit cytochrome P450 2A-dependent metabolic pathways They report that coumarin 7-

hydroxlation was significantly decreased with 7-hydroxycoumarin excretion delayed by up to 6 hours 13 volunteers were assessed for the effect of the grapefruit juice on their production of 7-hydroxycoumarin *in vivo*

Egan and O'Kennedy (1993a) developed a spectrofluorimetric microassay for the determination of 7-hydroxycoumarin in urine and plasma. The samples were initially analysed without sample clean-up The samples were then treated with β-glucuronidase, extracted into diethyl ether, evaporated to dryness and reconstituted into methanol. The linear range for detection of 7-hydroxycoumarin, before or after extraction, in urine and plasma was 0 5 -10 µg/ml and 10 - 100 µg/ml, respectively, and the limit of quantification was 0 5 µg/ml. The excitation wavelength chosen was 370 nm and emission was monitored at 450 nm. The assay was carried out in 96 well Dynatech microtitre plates

1 3 2. Total Luminescence Spectroscopy

Coumarin is added as a marker compound to separate rebated and unrebated kerosene for the purpose of customs excise duty (Shanahan *et al* , 1991) A quick method of analysis of the fuel oils is to convert the coumarin present into the potassium salt of *cis*-coumaric acid, and, furthermore, into the *trans* isomer by irradiation with UV light at 365 nm. This isomer is fluorescent and coumarin can be qualitatively determined Coumarin is quantified by measuring the fluorescence obtained by excitation at 365 nm and emission at 500 nm. Total luminescence spectra of the fuel samples are obtained by examining all the fluorescence spectra across a range of wavelengths (300 nm to 600 nm)

1 4 ANTIBODY-BASED IMMUNOANALYTICAL TECHNIQUES

The antibody-antigen interaction can be used as an analytical tool for selective and sensitive determinations (Tijssen, 1985, Killard *et al*, 1995) of drug, hormones etc The production of antibodies, their functions and their applications are reviewed by Killard *et al* (1995) The specificity of an antibody determines its suitability as a potential tool for sensitive, accurate analysis of coumarin and its derivatives Table 1 4 1 summarises the antibody based immunoanalytical techniques available for coumarin and 7-hydroxycoumarin determination discussed in the section below Chapter 5 gives an introduction to antibodies, to the antigen-antibody interaction and to an enzyme-linked immunosorbent assay (ELISA)

Method &Application	**Mode of Detection**	**Limit of Detection**	**Reference**
ELISA Analysis of Coumarin or 7-hydroxycoumarin - Antigen inhibition ELISA with anti-coumarin / 7-OHC antibody	OPD as substrate with detection at 405 nm pNPP as substrate with detection at 414 nm	0 5 µg/ml 40 ng/ml	Egan (1993) and Egan / O'Kennedy, (1993b) Reinartz *et al* (1996)
7-hydroxycoumarin-alkaline-phosphatase-bispecific antibody assay	pNPP as substrate with detection at 414 nm	6 ng/ml	Reinartz *et al* (1996)
Electroanalytical Analysis Direct analysis of 7-OHC Indirect analysis of 7-OHC	Electrochemistry of 7-OHC Electrochemistry of HRP	10 µM 24 µM	Dempsey *et al* (1993a) Deasy *et al* (1994)
Biospecific Interaction Analysis 7-hydroxycoumarin assay	Surface plasmon resonance	0 5 µg/ml	Keating *et al* (1995)

Table 1 4 1 Summary of the immunoanalytical methods of analysis of coumarins. Table of the methods of analysis, detection, application, limit of detection and reference.

1 4 1 Enzyme-linked immunosorbent assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) is an antibody-based method for the detection of drugs in body fluids (also see section 5 1 4 1) A large variation of assay designs have been used for the detection of antibodies and antigens (Catty and Raykundalia, 1989, Kemeny, 1991, Killard *et al*, 1995) Immunoassays can be divided into heterogenous and homogenous assays In a heterogenous assay, a separation step is required where the free antibody and antigen is separated from the solid phase bound antibody-antigen complex The quantifiable signal from the complex does not differ from the free entity and therefore without this separation, interference would occur In a homogenous assay, this separation step is not required The binding of antigen and antibody results in a change in the activity of the label and therefore this activity can be detected without the problems of interference An heterogenous immunoassay will have the following steps common to all variations

1 Immobilisation of antigen or antibody on a solid surface (usually a microtiter plate)
2 Incubation with a sample containing the corresponding antigen or antibody
3 Detection of specifically bound antibody or antigen by binding an enzyme-linked antibody or antigen, using a colourimetric reaction employing the enzyme

The method most frequently used for the quantification of haptens (e g drugs, hormones etc) is the antigen-inhibition ELISA (Egan and O'Kennedy, 1993b)

1 4 1 1 Determination of coumarin and 7-hydroxycoumarin by antigen inhibition enzyme-linked immunosorbent assay

Antigen inhibition ELISA has been used to detect both coumarin and 7-hydroxycoumarin in urine (Egan, 1993, Egan and O'Kennedy, 1993b, Reinartz and O'Kennedy, 1995) For the detection of coumarin (Egan, 1993), a coumarin-thyroglobulin-conjugate was immobilised on an ELISA microtiter plate Coumarin standards and unknowns (n=4) were incubated with purified polyclonal anti-bovine serum albumin-coumarin IgG antibodies raised in rabbits at 37 °C for 2 hours A competition occurs between the free coumarin in solution and the coumarin coated on the plate surface for the antibody Unbound antibody and free coumarin-antibody complexes are washed off leaving bound coumarin-antibody complexes behind on the plate Bound antibody was detected using HRP-labelled

secondary goat anti-rabbit IgG, with o-phenylene diamine (OPD) as the substrate The linear range of the assay is from 10-100 µg/ml. A similar protocol is followed for the determination of 7-hydroxycoumarin by ELISA (Egan and O'Kennedy, 1993b) The antibody was raised against BSA-7-hydroxycoumarin and screened with ovalbumin-7-hydroxycoumarin The linear range of this assay is from 5-100 µg/ml with a limit of detection of 0 5 µg/ml

A similar system, employing a BSA-7-hydroxycoumarin-conjugate immobilised at the microtiter plate surface, an anti-7-hydroxycoumarin IgG raised in rabbits, and alkaline phosphatase-labelled goat anti-rabbit IgG as detection antibody with paranitrophenol phosphate (pNPP) as substrate, was found to be more sensitive (Reinartz and O'Kennedy, 1996) The linear range was from 50 ng/ml to 5 µg/ml, with a limit of detection of 40 ng/ml

1 4 1 2 Antigen inhibition ELISA employing a bispecific antibody

Bispecific antibodies are antibodies, which, in contrast to natural antibodies, can bind to two different antigens simultaneously Bispecific antibodies have been produced chemically by crosslinking whole antibodies (bispecific multivalent antibodies, bsMab) or fragments (Fab'- bs(Fab')$_2$), biologically using hybridoma technology, or through genetic engineering Their production, potential, and advantages in enzyme immunoassays are reviewed by Nolan and O'Kennedy (1992)

bsMab's can be rapidly synthesised by the heterobifunctional cross-linkage of two parental antibodies (Cook and Wood, 1994) Such a bispecific antibody was produced from an anti-thyroglobulin-7-hydroxycoumarin and an anti-alkaline phosphatase antibody, using the method of Cook and Wood (1994) The product was used to develop a one-step antigen inhibition ELISA for the detection of 7-hydroxycoumarin in urine (Reinartz and O'Kennedy, 1995) The plate was coated with BSA-7-hydroxycoumarin 25 µl of 7-hydroxycoumarin standard or unknown, 50 µl of bsMab at its working dilution, and 25 µl of alkaline phosphatase, at a well concentration of 100 µg/ml, were added and incubated

for 90 min at 37 °C Colour was developed with paranitrophenol phosphate as substrate and absorbance at 414 nm.

Free 7-hydroxycoumarin was determined in urine using this method, and had a linear range between 20 ng/ml and 4 µg/ml Total 7-hydroxycoumarin determination required the deconjugation of 7-hydroxycoumarin-glucuronide by the addition of β-glucuronidase All samples were diluted 1 10 in 60 mM PBS-Tween 20 and the samples were analysed as above The linear range for the determination of total 7-hydroxycoumarin was from 100 ng/ml and 1 µg/ml with a limit of detection of 30 ng/ml. Accuracy and precision was determined by spiking 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide into control urine Samples were also analysed after sample clean-up Samples were extracted into diethyl ether, evaporated off and reconstituted into PBS-Tween 20 The limit of detection was 6 ng/ml with a linear range between 6 ng/ml and 800 ng/ml. Samples were analysed by this method and by HPLC (Egan and O'Kennedy, 1992) There was no statistical difference between the different methods

1.4.2 Immunoelectroanalytical Analysis

Dempsey *et al* (1993a), Deasy *et al* (1994) and Lu *et al* (1996) have investigated the use of anti-7-hydroxycoumarin antibodies for the determination of 7-hydroxycoumarin A biosensor based on the immobilisation of anti-7-hydroxycoumarin antibodies at the surface of a glassy carbon electrode behind a permeable cellulose dialysis membrane was developed by Dempsey *et al* (1993a) The electrochemical behaviour of 7-hydroxycoumarin at a bare glassy carbon electrode is documented (Dempsey *et al*, 1993b) However, when the antibody is present at the electrode surface there is a 90% decrease in 7-hydroxycoumarin electrochemical response The binding of the 7-hydroxycoumarin to the antibody is believed to occur at its electroactive site which would explain the decrease in the response at the electrode surface Anodic detection of the oxidised product was achieved at 0 6 V using DC amperometry The limit of detection was 10 µM Antibody-antigen kinetics were also investigated A first-order reaction was observed with k= 0 0329 mA/min

An enzyme-linked electrochemical competitive immunoassay was developed by Deasy *et al* (1994) The anti-7-hydroxycoumarin antibodies were labelled with horseradish peroxidase The electrochemical character of horseradish peroxidase is utilised for the indirect determination of 7-hydroxycoumarin The glassy carbon surface was coated with thyroglobulin-7-hydroxycoumarin Different concentrations of 7-hydroxycoumarin were added together with antibodies raised, in rabbits, against bovine serum albumin-7-hydroxycoumarin A competition, similar to the competitive ELISA above occurs, between free 7-hydroxycoumarin and 7-hydroxycoumarin at the electrode surface After antibody-antigen interaction all unbound solutions are removed by washing leaving labelled antibody at the surface The electrochemical activity of the horseradish peroxidase on the labelled antibody at the electrode surface is then determined The current is measured after the addition of 1 mM hydrogen peroxide (enzyme substrate), in the presence of 2 mM hydroquinone The current output was inversely proportional to the concentration of free 7-hydroxycoumarin in solution The limit of detection was 24 μM and 7-hydroxycoumarin concentrations could be determined up to 1 mM

Lu *et al* (1996) developed a regeneratable enzyme immunosensor for 7-hydroxycoumarin based on electrochemical detection BSA-7-hydroxycoumarin is chemically immobilised at the surface of a glassy carbon electrode A competition is set-up between free 7-hydroxycoumarin in solution and BSA-7-hydroxycoumarin coated at the electrode surface for binding of a horseradish peroxidase labelled anti-7-hydroxycoumarin antibody The reduction of hydrogen peroxide in the presence of potassium ferrocyanide (a mediator in the reaction) was used for the determination of 7-hydroxycoumarin After binding of the antibody to the BSA-7-hydroxycoumarin conjugate the excess antibody and free 7-hydroxycoumarin were removed and the reduction of hydrogen peroxide is determined by DC voltammetry at a potential at 0 0 V vs Ag/AgCl. The surface of the electrode was regenerated by immersing the electrode in 10 mM HCl to dissociate the antibody-hapten complex The regenerated electrode was then washed with phosphate buffered saline, ready for the next analysis

1 4 3 Biospecific Interaction Analysis

A competitive immunoassay for the analysis of 7-hydroxycoumarin in biological samples has been developed, based on a commercial biosensor system - the BIAcore™ from Pharmacia Biosensor (Keating and O'Kennedy, 1995) The BIAcore (real-time Biospecific Interaction Analysis) makes use of the phenomenon of surface plasmon resonance (SPR) to directly monitor biomolecular binding events without the need to label interactants (Fagerstam and O'Shannessy, 1993)

The BIAcore sensor system utilises a combination of optical and continuous-flow technologies to facilitate label-free interaction analysis Biomolecular binding is studied by immobilising one of the components on the surface of a specialised sensor chip within the biosensor, and allowing the other to flow over the surface in solution Mass changes at the biosensor chip surface due to the binding of two or more components causes a change in resonance angle directly proportional to the mass change An indirect competitive immunoassay was developed for the determination of 7-hydroxycoumarin in serum (Keating and O'Kennedy, 1995)

The assay consists of the following steps

1 Immobilisation of 7-hydroxycoumarin-protein conjugate on sensor surface
2 Samples/standards mixed with β-glucuronidase to deconjugate 7-hydroxycoumarin-glucuronide to 7-hydroxycoumarin
3 Incubation for 2 hours at 37 °C
4 Addition of antibody (0 5 mg/ml) to samples/standards
5 Incubation for 5 minutes
6 Injection over sensor chip surface
7 Regeneration of sensor surface

A 7-hydroxycoumarin-bovine serum albumin (7-OHC-BSA) is first immobilised on the sensor chip surface This is done by derivatisation with N-hydroxysuccinimide (NHS), mediated by N-ethyl-N'-(dimethylaminopropyl)carbodiimide hydrochloride (EDC) The immobilisation procedure is highly reproducible, resulting in the covalent binding of about 10,000 response units (RU) of 7-OHC-BSA The antibody used in the assay is a rabbit polyclonal anti-7-hydroxycoumarin purified from serum. 20ml of antibody-sample mixture

is injected The 7-OHC-BSA on the sensor surface and the 7-OHC in the sample compete for antibody binding The resulting signal - due to unbound antibody binding to the drug-protein conjugate immobilised on the sensor chip - is inversely proportional to the amount of 7-hydroxycoumarin present in the sample

A standard curve of concentration versus relative signal is prepared In this assay, regeneration of the 7-OHC-BSA surface is achieved by injection of a 1 minute pulse of 5mM NaOH, a pulse of 20mM HCl, and another pulse of 5 mM NaOH Following the regeneration of the surface the next sample or standard is then analysed All the steps in the assay, including the addition of, and incubation of, both enzyme and antibody, are automated and carried out on the BIAcore The assay is suitable for the analysis of 7-hydroxycoumarin in neat serum and plasma, without the need for extraction of the drug Neat urine samples may also be analysed, although variations in salt content and pH are problematic The range of the assay is from 0 5 µg/ml to 80 µg/ml

1.5 ELECTROANALYTICAL AND OTHER CHROMATOGRAPHIC TECHNIQUES

1 5.1 Electroanalytical Analysis

Cyclic voltammetry was carried out on 7-hydroxycoumarin prepared in phosphate buffer at an electrochemically pre-treated glassy carbon electrode (Dempsey *et al*, 1993b) The potential was cycled between -1 5 V and +1 5 V From this the electrochemical characteristics of 7-hydroxycoumarin were found An electroanalytical assay was then developed using differential pulse voltammetry as the method of detection Samples of 7-hydoxycoumarin were prepared in urine, treated with β-glucuronidase and extracted according to Egan and O'Kennedy (1992) and reconstituted into methanol The sample was then analysed by determining the electrochemical response at an anodic detection at 0 66 V The method was found to be linear between 0 and 15 mM The method was also applied to the determination of 7-hydroxycoumarin in patient urine samples

Differential pulse voltammetry was also used to analyse 7-hydroxycoumarin and 7-amino-4-methyl-coumarin in micellar and emulsified media (Carrazon *et al*, 1989) Carrazon *et al* (1989) analysed the coumarins by differential pulse voltammetry (DPV) and by linear sweep voltammetry (LSV) with a rotating and stationary glassy carbon electrode (GCE) The detection limits for 7-hydroxycoumarin were DPV 0 87 µM for a stationary GCE, and 0 28 µM for the rotating GCE The limit of detection for LSV at a stationary GCE was 4 9 µM, and 0 63 µM for a rotating GCE The above values were determined in a micellar solution (0 08% Triton X-405, 0 2 M acetate buffer, pH 4 8) The samples were prepared in micellar solution or as emulsions to avoid the use of organic solvents and to allow their dissolution into aqueous media.

Polarography was used to determine coumarin in sweet clover and in preparation FIBS (Orlov, 1988, and Orlov *et al*, 1992) Coumarin from samples of white and yellow sweet clover was extracted into boiling ethanol. After distilling off the ethanol a 1 ml reconstituted sample, in ethanol, was added to 4 ml of ethanol and 1 ml of a 1 % solution of tetraethylammonium iodide Polarography was carried out with a dropping mercury electrode Coumarin is used as a stabiliser in the injection solution of FIBS and polarography is used as a selective detector to separate it from cinnamic acid, also present as a stabiliser Samples of coumarin were analysed by polarography with cathodic polarisation in the interval 1 20 V - 2 00 V ($E_{1/2}$ = 1 67 V)

1.5 2 Other Chromatographic Techniques

Planar chromatography (Betti *et al*, 1993), centrifugal partition chromatography (Marston *et al*, 1993), and high speed countercurrent chromatography (Schaufelberger, 1989) have all been used for the determination of coumarins Betti *et al* (1993) report on a planar chromatography method utilising a series of different stationary phases and mobile phases in order to discover the best separation technique for the different compounds of interest including umbelliferone, quercetin, and psoralene The stationary phases include C_{18}, cyano-, silica, phenyl, amino, diol, and C_2 Mobile phases include mixtures of methanol, acetic acid, acetone, toluene, ethyl acetate, or formic acid

Centrifugal liquid-liquid partition chromatography (Marston *et al*, 1988) relies on the separation of compounds between two immiscible phases and the application of a centrifugal force during that separation Samples of umbelliferone, herniarin, and scopoletin were separated in a light petroleum-ethyl acetate-methanol-water system Fractions are collected and analysed by UV detection A chloroform-methanol-water mobile phase was used for the separation of coumarins by high-speed analytical counter-current chromatography (Schaufelberger, 1989) with photodiode array detection

Summary

The analytical approaches used to detect coumarin and its derivatives are many and varied The choice of method is dependent on the particular coumarin of interest, and the matrix in which it is a component The ideal method is obviously one which will involve minimal sample preparation, short analysis time, be relatively inexpensive, and have excellent accuracy and precision for a variety of applications Separation and detection of the coumarin in its particular matrix, is dependent on many factors These methods are dependent on whether it fluoresces or not, whether it has a strong UV absorbance or not, and whether there are antibodies available against that particular coumarin Migration on CE, retention on a HPLC column, or its volatility for GC analysis will vary for each coumarin and determine its resolution from other species present The selectivity of antibody-based systems may be needed to isolate the analyte of interest from complex endogenous species present that may interfere with analysis However, it is now clear that there are a variety of methods now available with good analytical characteristics which may be applied to complex matrices and these may be modified to suit the requirements of a particular coumarin or coumarins or specific applications

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

2 1 REAGENTS AND EQUIPMENT

METHODS

2.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

2 2 1 Preparation of HPLC mobile phases

2.2 2 Preparation of samples and standards for HPLC analysis

2 2 2 1 The collection and preparation of urine, plasma and serum

2 2 2 2 The preparation of samples for analysis

2.2.3 HPLC separation parameters

2 2 3 1 Isocratic method

2 2 3 2 Gradient method

2 2 3.3 Analysis of proteins by HPLC

2 3 CAPILLARY ELECTROPHORESIS (CE)

2 3.1. Preparation of CE electrolyte solutions

2.3.2. Preparation of samples and standards for analysis by CE

2 3 2 1 Deconjugation and extraction

2 5. PROTEIN-DRUG CONJUGATES AND PRODUCTION OF AN ANTI-7-HYDROXYCOUMARIN ANTIBODY

2.5 1 Production of protein-drug conjugates

2.5 2 Immunisation protocol

2 5 3 Purification of Antibody

2 5.4. Enzyme linked immunosorbent assay

MATERIALS

2.1 REAGENTS AND EQUIPMENT

A list of the equipment and reagents used and their suppliers are given in Table 2 1 1 and Table 2 1 2 , respectively All reagents used were of analytical grade All organic solvents used were of HPLC grade The water used for all buffers and mobile phases was of Ultrapure Grade

Equipment	Suppliers
Nicolet I R Spectrometer	Nicolet Instr Corp , Madison, WI, USA
Heraeus Sepatech Biofuge 13, and Heraues Labofuge GL, Titretek Twinreader-plus	Medlabs, Dublin Ireland
System Gold solvent module 126, detector module 166, photodiode array 168, autosampler 507, P/ACE System 2050 and System 5500, Beckman L8-70M Ultracentrifuge,	Beckman Instruments Limited, Fullerton, CA, USA
400 MHz NMR spectrometer	Brucker, Coventry, England
UV-160A Spectrometer	Shimadzu Corp , Kyoto, Japan
3015 pH meter	Jenway Ltd , Essex, England
Hetosicc, Hetofrig and Hetovac freeze dryer	Heto Lab equipment A/S, Allerod, Denmark
Ultra-pure water	Millipore, Bedford, MA, USA
Ultra-Turrax, Kika-Werk Homogeniser	Lennox, Dublin, Ireland

Table 2 1 1 Equipment and Suppliers

Reagent	Company
Bovine serum albumin, thyroglobulin, o-phenylene diamine (OPDA), ammonium sulphate ($(NH_4)_2SO_4$), sodium chloride, deoxycholic acid, 7-hydroxycoumarin, sodium and potassium hydrogen phosphate ($NaHPO_4$, $KHPO_4$), sodium and potassium phosphate (Na_2PO_4, K_2PO_4), nicotine adenine dihydrogen phosphate, goat anti-rabbit peroxidase labelled antibody, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, sucrose, sodium acetate, UDP-glucuronic acid, 4-hydroxycoumarin, magnesium chloride, saccharic acid 1,4-lactone, β-glucuronidase, dithiothreitol, Freunds complete and incomplete adjuvant, protein molecular weight standards	Sigma Chemical Co , St Louis, MO, USA
Trichloroacetic acid, tris-HCl, Hydrogen peroxide	BDH Chemicals Ltd , Poole, England, Carl Stuart, Dublin, Ireland
HPLC grade methanol, diethyl ether, acetonitrile, Petroleum-ether	Labscan, Dublin, Ireland
1 6 ml plastic eppindorf tubes, 10 ml NH_4-Heparin AH/10 treated tubes for blood collection	Sarstedt, Wexford, Ireland
Sodium hydroxide, Tween 20, hydrochloric acid, acetic acid, Silica TLC plates	Riedel de-Haen, Hannover, Germany
Coumarin, 7-hydroxycoumarin-glucuronide	Schaper and Brummer, Salzgitter, Germany
1 1 ml screw cap HPLC vials, PTFE septa	Carl Stuart, Dublin, Ireland
Untreated silica capillaries, 50 ml polyallomer quick-seal centrifuge tubes	Beckman Instruments Limited, Fullerton, CA, USA
Phosphate Buffered saline tablets	Unipath, Hampshire, England
Bicinchoninic acid (BCA) assay	Pierce and Warringer, Cheshire, England
NUNC Maxisorb plates	NUNC,Kamstrup DK, Roskilde, Denmark
7-amino-4-methyl coumarin	Aldrich, Dorset, England
Phenomenex Bondclone 10 C18 HPLC column,	Phenomenex, Macclesfield, England
10 ml screw cap untreated blood tubes, 12 mm X 75 mm glass tubes	Medlabs, Dublin Ireland

Table 2 1 2 Reagents and Suppliers

2 2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

All HPLC analysis was carried out on system Gold HPLC apparatus (see Table 2 1) The system consisted of a dual pump/solvent delivery system and solvent mixer [module 126], an autosampler injection system [module 507], a UV detector [module 166], a solvent waste bottle and data processing unit (computer- Dell Dimension 486) -see Figure 4 0 1 The system was controlled by System Gold™ software (version 8 0)

2 2.1 Preparation of HPLC mobile phase

The mobile phases utilised for the separation and determination of coumarins in the various areas studied consisted of a water methanol acetic acid mix Analysis of serum samples from a Phase I drug trial of 7-hydroxycoumarin used the method of Egan and O'Kennedy (1992) Separation was based on an isocratic method with a water methanol acetic acid mobile phase (700 300 2 v/v/v) The method developed for the determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide after *in vivo* and *in vitro* metabolism of coumarin or 7-hydroxycoumarin used gradient elution The mobile phases were (A) water methanol acetic acid (950 50 2 v/v/v) and (B) methanol (100 %) The mobile phase used for the analysis of proteins was 0 1 M phosphate buffer, pH 7 4 The mobile phases prepared were mixed thoroughly and degassed by sonication for 20 min (organic solvents) or filtered and degassed under vacuum (phosphate buffer)

2.2 2 Preparation of samples for HPLC analysis

2 2 2 1 The collection and preparation of serum, urine and plasma

2 2 2 1 1 The preparation of stripped serum

Control serum was obtained from St James' Hospital (Dublin, Ireland) For every 200 ml of serum 10 g of activated charcoal was added, mixed and incubated at 45 °C for 20 min This was then removed and centrifuged at 3000 rpm for 15 min and the

supernatant was collected For each ml of supernatant, 50 mg of activated charcoal was added and incubated in the water bath for a further 20 min and then centrifuged This was repeated a further two times Following this the supernatant was filtered through 0 45 μm disposable sterile filters and stored at -20 °C until required

2 2 2 1 2 Patient serum collection and preparation

59 patients were treated orally with 7-hydroxycoumarin as part of a Phase I clinical trial 29 male and 30 female patients were diagnosed with a range of advanced malignancies (Table 2 2), and their ages varied from 27 to 83 years (see section 4 3)

Diagnosis	No. of Patients
Renal cell carcinoma	15
Colon	15
Prostate	8
Carcinoid	6
Non-small cell	5
Hepatoma	2
Small cell carcinoma	1
Adrenal	1
Parotid	1
Gallbladder	1
Rectal	1
Neurosarcoma	1
Bronchial carcinoma	1
Bladder	1

Table 2 2 Patient diagnosis and the number of patients diagnosed with a particular malignancy Serum samples were obtained from the patients as part of the metabolism study trial of 7-hydroxycoumarin in patients with advanced malignancies

The range of doses investigated were from 100 mg to 7,000 mg (each of 3 patients were administered 100 mg, 300 mg, 500 mg, 700 mg, 900 mg, 1200 mg, 1500 mg, 2000 mg, 2500 mg, 3000 mg, 3500 mg, 4000 mg, 4500 mg, 5000 mg, 5500 mg (2 patients only), 6000 mg, and each of 6 patients were administered 6500 mg and 7000 mg respectively) Blood samples were taken pre-administration and at 5, 15, 30, 45, 60 min and 2, 4, 8, and 24 hr after drug administration The drug was readministered

14 days later and samples were taken at the above times again The blood was allowed to clot and stored immediately, after the removal of the clot, at -80 °C until analysis

2 2 2 1 3 Control urine and plasma collection and preparation

Blank urine and plasma was obtained from healthy volunteers who had not been treated with coumarin and / or 7-hydroxycoumarin The urine was collected into a sterile glass bottle, centrifuged and filtered through a 0 22 µm sterile filter Control plasma was kindly donated by Professor R D Thornes

2 2 2 1 4 Volunteer urine and plasma samples collection and preparation

In two different studies volunteers were administered 100 mg and 250 mg of coumarin respectively Urine samples were taken before the administration of coumarin and at 2, 6, 10, 14, and 24 hr after administration The samples were collected in sterile 500 ml bottles and the urinary volumes were recorded Before analysis the urine samples were centrifuged at 3000 rpm for 5 min to remove excess precipitated salts Blood samples were taken before the administration of 250 mg of coumarin and at 1 hr and 4 hr after administration The blood was added into 10 ml screw cap tubes treated with NH_4-Heparin to prevent clotting The samples were centrifuged at 3000 rpm for 20 min and the plasma removed Both urine and plasma control and samples were stored at -20 °C until required

2 2 2 2 The preparation of samples for analysis

2 2 2 2 1 Determination of free and total 7-hydroxycoumarin by HPLC

Deconjugation and extraction of 7-hydroxycoumarin

Serial dilutions of 7-hydroxycoumarin were prepared in deionised water from a stock 1 mg/ml solution prepared in 10 % methanol 90 % water Into a 10 ml sterile blood tube, 0 1 ml volume of 7-hydroxycoumarin standard was added to 0 9 ml of control serum or urine giving a series of calibration standards between 0 and 100 µg/ml (0, 0 5, 1 0, 5 0, 10 0, 20 0, 50 0, 80 0 and 100 0 µg/ml, respectively) Into a 10 ml sterile blood tube 1 ml samples of the patient serum or volunteer urine was added To both standard and unknown samples 0 05 ml of a 1 mg/ml internal standard (7-amino-4-methyl-coumarin, prepared in 100 % methanol) solution was added and vortex mixed

To liberate conjugated 7-hydroxycoumarin a 1 0 ml β-glucuronidase solution was added (5000 units/ml prepared in 1M sodium acetate, pH 5 0) , gently mixed, and incubated for 30 min at 37 °C After incubation the 7-hydroxycoumarin present was extracted into diethyl ether 3 5 ml of diethyl ether was added into each tube and rotary mixed for 10 min The tubes were then centrifuged at 2000 rpm for 10 min 1 8 ml of the organic layer are removed into a 75 mm x 12 mm glass tube and evaporated to dryness at 60 °C The sample was then reconstituted into 0 2 ml of methanol, vortex mixed for 10 sec and centrifuged at 2,000 rpm for 2 min before analysis by HPLC 160 µl of the reconstituted sample was added to a 1 1 ml screw cap vial before analysis (see section 4 0) Samples were analysed by the isocratic method (see section 2 2 3 1)

2 2 2 2 2 Determination of total 7-hydroxycoumarin by HPLC

Deconjugation and protein precipitation

Urine samples

In a 1 6 ml eppendorf tube, 0 5 ml of β-glucuronidase (5000 units/ml in 1 0 M sodium acetate, pH 5 0) was added to 0 5 ml of urine sample or standard and incubated at 37 °C for 30 min 100 µl of a 20 % (w/v) solution of trichloroacetic acid was then added, to precipitate the protein solution, and the samples were mixed and centrifuged at 13,000 rpm for 5 minutes 10 µl of a 1 mg/ml 4-hydroxycoumarin, prepared in 100 % methanol, (the internal standard) was added to 190 µl of supernatant and vortex mixed Samples were analysed by the gradient method (see section 2 2 3 2)

Plasma samples

To 100 µl of 7-hydroxycoumarin or 7-hydroxycoumarin-glucuronide standard, prepared in blank plasma, or 100 µl of plasma sample, 100 µl of β-glucuronidase (5000 units/ml in 0 5 M sodium acetate, pH 5 0) was added and incubated at 37 °C for 60 min 20 µl of a 20 % (w/v) solution of trichloroacetic acid was added, mixed and centrifuged at 13000 rpm for 5 min To 150 µl of supernatant 10 µl of a 1 mg/ml 4-

hydroxycoumarin, prepared in 100 % methanol, (the internal standard) was added and vortex mixed Samples were analysed by the gradient method (see section 2 2 3 2)

2 2 2 3 Determination of coumarin, free 7-hydroxycoumarin and conjugated 7-hydroxycoumarin by HPLC

Preparation of urine, serum and plasma samples for direct analysis of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide

Preparation of 7-hydroxycoumarin-glucuronide

Rabbits were fed 7-hydroxycoumarin and the urine of the animals was collected The glucuronide was purified by liquid chromatography The separation column was filled with DEAE-cellulose MN 2100 (Macherey & Nagel) and the sample applied For elution ammoniumformate buffer, pH 9 0, was used After drying the 7-hydroxycoumarin-glucuronide was extracted several times with methanol The resulting 7-hydroxycoumarin-glucuronide was recrystallised from water and ethanol/water (The protocol and compound was kindly supplied by Dr M Tegtmeier, Schaper & Brummer, Salzgitter, Germany)

Urine samples

A series of 7-hydroxycoumarin, coumarin and 7-hydroxycoumarin-glucuronide standards were prepared in water from 1 mg/ml stock solutions (0, 20, 50, 100, 200, 500, 800 and 1000 µg/ml respectively) To 140 µl of blank urine, 20 µl each standard and 10 µl of internal standard (1 mg/ml 4-hydroxycoumarin, prepared in 100 % methanol) was added and vortex mixed To 200 µl of sample 10 µl of the internal standard was added Samples were analysed by the gradient method (see section 2 2 3 2)

Serum and plasma samples

To 140 µl of control plasma or serum 20 µl of each of the standards was added and vortex mixed (the standards prepared were 0, 1, 5, 10, 20, 50, 80 and 100 µg/ml) To 200 µl of sample or the standard solution 40 µl of a 20 % solution of trichloroacetic

acid was added and vortex mixed This was then centrifuged at 13,000 rpm for 10 min To 190 µl of the supernatant 10 µl of the internal standard (1 mg/ml 4-hydroxycoumarin, prepared in 100 % methanol) was added, vortex mixed and then analysed by the gradient HPLC method (see section 2 2 3 2)

Calculation of total 7-hydroxycoumarin content in serum

It was assumed that 99 % of the 7-hydroxycoumarin in the sample was present as 7-hydroxycoumarin-glucuronide and from this the total 7-hydroxycoumarin content was calculated by multiplying the molar ratio (0 48, i e Molecular Weight 7-OHC / Molecular weight of 7-OHCG - 162 1/338 1 x 100/1) by the 7-hydroxycoumarin-glucuronide concentration

2 2 2 4 Determination of 7-hydroxycoumarin-glucuronide after the in vitro metabolism of 7-hydroxycoumarin

Preparation of samples and standards for the in vitro metabolism study of 7-hydroxycoumarin

Component	Stock solution concentration	Volume (µl)	Final concentration
7-hydroxycoumarin	6 2 mM	500	0 77 mM
UDPGT enzyme preparation	4 mg/ml	1000	1 mg/ml
Magnesium Chloride	1 M	25	6 25 mM
Saccharic acid 1,4-lactone	50 mM	500	6 25 mM
UDP-glucuronic acid	10 mM	500	1 25 mM
Absolute Ethanol	-	125	-
Tris-HCl (pH 7 4)	1 M	500	125 mM
Deionised water	-	850	-

Table 2 2 2 2 1 Concentrations and volumes of the components of the incubation mixture used in the in vitro production of 7-hydroxycoumarin

The mixture (Table 2 2 2 2 1) was prepared for the *in vitro* production of 7-hydroxycoumarin-glucuronide 7-hydroxycoumarin was prepared by diluting a 6 2 mM (1 mg/ml) stock solution (prepared in 10 % ethanol water) with water All other

components were prepared in water The reaction was carried out at 37 °C in an open 10 ml glass tube and initiated by the addition of the enzyme solution 200 µl of the reaction mixture was removed at specific time intervals and added to 40 µl of a 20 % (w/v) solution of trichloroacetic acid, mixed and centrifuged at 13,000 rpm for 5 min before analysis by HPLC

A series of 7-hydroxycoumarin-glucuronide (0 - 1000 µg/ml) standards were prepared in water from a 1 mg/ml stock solution A standard curve was prepared as follows 20 µl 7-OHCG standard, 40 µl enzyme solution, 40 µl water, 100 µl tris-HCl, and 40 µl trichloroacetic acid were added together and centrifuged at 13000 rpm for 5 min The total volume was exactly the same as that of the sample removed from the metabolic solution 190 µl of the sample or standard supernatant were added to 10 µl of a 1 mg/ml solution of the internal standard (4-hydroxycoumarin) in a 1 1 ml sample vial for analysis on HPLC Samples were analysed by the gradient method (see section 2 2 3 2)

2.2.2.2 5 Determination of proteins by HPLC

Qualitative determination of proteins

A 1 mg/ml solution of the protein-drug conjugates and controls (see section 2 5 1) were prepared in water The purified antibody (section 2 5 3), prepared in 0 14 M PBS, pH 7 4 was diluted to 1 mg/ml

2.2.3 HPLC separation parameters

The HPLC system consisted of a System Gold solvent module 126, detector module 166, photodiode array detector 168, and autosampler module 507 All of the components were controlled by System Gold Software Reverse-phase HPLC separations were carried on a 300 mm X 3 9 mm Phenomenex (Macclesfield, England) Bondclone 10 C_{18} column Protein samples were separated using a Protein Pak (Millipore) SW 300 column with a particle diameter of 10 µm.

2 2 3 1 Isocratic method

The mobile phase was a 700 300 2 water methanol acetic acid (v/v/v) solution The flow rate was 2 ml/min Detection was at 320 nm 20 µl samples were injected onto the column for analysis

2 2 3 2 Gradient method

Solvent A was a 950 50 2, water methanol acetic acid (v/v/v) solution Solvent B was 100 % methanol The flow rate was 1 ml/min Detection was at 320 nm The 1 ml/min gradient was as follows

0 min -14 min Solvent A (100%) → Solvent A (50 %) / Solvent B (50 %), 14 min- 22 min Solvent A (50 %) / Solvent B (50 %), 22 min-23 min Solvent A (50 %) / Solvent B (50 %) → Solvent A (100 %), 23 min- 32 min Solvent A(100 %)

Detection was at 320 nm 20 µl samples were injected onto the column for analysis Figure 2 2 3 2 1 shows the gradient used for the separation of coumarin, 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and 4-hydroxycoumarin

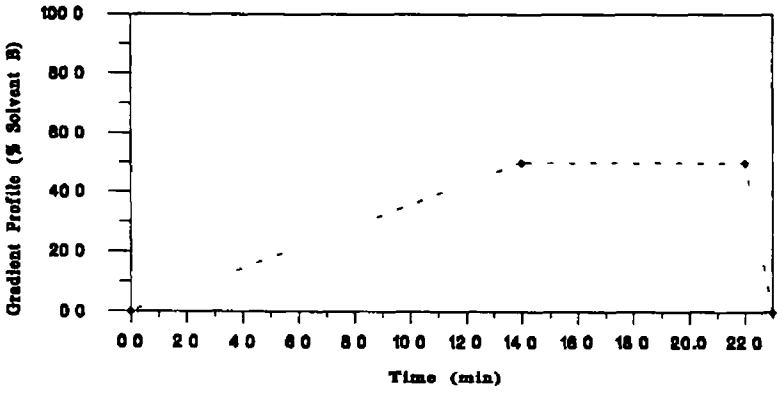


Figure 2 2 3 2 1 Graphical representation of the HPLC gradient elution profile used for the separation of coumarin, 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide, and 4-hydroxycoumarin (see section 2.2 3 2)

2 2 3 3 Analysis of proteins by HPLC

Protein-drug conjugates

The mobile phase used for the separation of the BSA- and thyroglobulin-7-hydroxycoumarin conjugates was 100 % ultra-pure water The mobile phase was filtered through a 0 45 µm filter and degassed by sonication for 20 min The flow rate was 0 5 ml/min, with photo diode array detection

Antibody Analysis

The purified antibody was separated by HPLC with the above mobile phase or with 0 1 M phosphate buffer, pH 7 4

2 3 CAPILLARY ELECTROPHORESIS

2 3 1 Preparation of CE electrolyte solutions

All buffers were prepared with deionised water (resistivity of 18 MΩ), and HPLC grade organic solvents

Phosphate Buffer

The various phosphate buffers used were prepared by mixing the acid and base salts (both prepared at the molarity required) of dipotassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) or the equivalent sodium salts until the required pH is achieved The solution was then sonicated for 5 min and filtered through a 0 22 µm sterile filter

90 % (100 mM phosphate Buffer / 11 mM deoxycholic acid (sodium salt)) 10 % acetonitrile

100 mM phosphate buffer was prepared in water and the 11 mM deoxycholic acid (sodium salt) was then dissolved in this buffer and thoroughly mixed To 18 ml of the viscous solution 2 ml of acetonitrile was then added, vortex mixed and filtered through a 0 22 µm sterile filter

2 3 2 Preparation of samples and standards for analysis by CE.

2 3 2 1 Deconjugation and extraction

The urine and serum samples and standards were prepared as outlined under Method 1 in the preparation of samples for HPLC analysis above However, the samples were reconstituted into phosphate buffer instead of methanol before analysis

2 3 2 2 Preparation of urine samples and standards for direct analysis of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine

7-hydroxycoumarin standards were prepared from a 1 mg/ml stock solution prepared in 20 80 (v/v) methanol deionised water 7-hydroxycoumarin-glucuronide standards were also prepared from a 1 mg/ml stock solution prepared in deionised water Dilutions (20-800 µg/ml) were then prepared in deionised water and 10 µl of each standard was spiked into 80 µl of urine Blank urine was also used, where necessary, for the dilution of samples with unknown metabolite levels to bring their values within the linear range of the assay The samples and standards were centrifuged at 13,000 rpm for 5 min before analysis

2 3 2 3 Microsomal in vitro metabolism of coumarin

Incubation solution

The incubation solution consisted of an NADPH regeneration system, coumarin and a liver microsomal preparation (Table 2 3 2 1) The concentrations of the NADPH-generating system in all incubations were 4 units/ml glucose-6-phosphate-dehydrogenase, 2 mg/ml D-glucose-6-phosphate, and 1 2 mM $NADP^+$ This was higher than that required to maintain maximum metabolic rates under the chosen *in vitro* conditions

Component	Stock Concentration	Volume added
Coumarin	0 21 mM	195 µl
D-glucose-6-phosphate	50 mg/ml	10 µl
D-glucose-6-phosphate-dehydrogenase	200 units/ml	5 µl
$NADP^+$	20 mM	15 µl
Microsomal suspension	various	25 µl

Table 2.3.2.1. Components of the incubation mixture for studying the in vitro metabolism of coumarin by CE

The coumarin (substrate) concentration in the incubation solutions was always 0 16 mM The components (Table 2 3 2 1) were mixed together in an 1 6 ml eppendorf tube to give final concentrations as above The reaction was started by the addition of 25 µl of microsomal suspension Following mixing the open Eppendorf tube was transferred immediately into the incubation bath (37 °C), and a 20 µl sample was taken and analysed The metabolite formation was monitored over a two hour period, taking samples at 0, 7, 14, 20, 30, 45, 60, 90, and 120 minutes, respectively In the case of analysis by HPLC, the incubation was first stopped by the addition of 50 µl of ice-cold trichloroacetic acid (20% w/v) and centrifuged at 13,000 rpm for 5 min before injection The HPLC analysis was carried out according to the method Egan and O'Kennedy (1992)

Preparation of standard curve

Standards were prepared by spiking 2 µl of 7-hydroxycoumarin and 4 µl of microsome preparation in 34 µl buffer The linear range was (0 - 308 5 µM, 0 - 50 µg/ml) with a limit of quantification of 6 17 µM (1 µg/ml) The concentration of 7-hydroxycoumarin produced by the microsomes was calculated from a plot of absorbance versus concentration of 7-hydroxycoumarin Standard curves were prepared in each of the microsomal preparations on the day on which the assay was carried out to allow for any day to day variations

2 3 2 3 1 NADP⁺ / NADPH Ratio Study

For this study, the concentration of glucose-6-phosphate-dehydrogenase was reduced to 0 1 units/ml The remainder of the incubation mixture was as above The study was carried out in the presence and in the absence of coumarin, or the microsomal preparation

2 3 2 4 The in vitro glucuronidation of 7-hydroxycoumarin

The incubation solution consisted of 8 components (Table 2 3 2 4 1) The principal components for the production of 7-hydroxycoumarin-glucuronide are 7-

hydroxycoumarin, the enzymatic solution containing the UDP-glucuronyl transferase, and UDP-glucuronic acid A 1 mg/ml solution of 7-hydroxycoumarin was prepared in 10 % ethanol (Merck, Darmstadt, Germany) 90% deionised water All other solutions were prepared in deionised water

Component	Stock solution concentration	Volume µl	Final concentration
7-hydroxycoumarin	6 2 mM	200	0 77 mM
UDPGT enzyme preparation	4 mg/ml	400	1 mg/ml
Magnesium Chloride	1 M	10	6 25 mM
Saccharic acid 1,4-lactone	50 mM	200	6 25 mM
UDP-glucuronic acid	10 mM	200	1 25 mM
Absolute Ethanol	-	50	-
Tris-HCl (pH 7 4)	1 M	200	125 mM
Deionised water	-	340	-

Table 2 3 2 4 1 Concentrations and volumes of the components of the incubation mixture used in the in vitro production of 7-hydroxycoumarin-glucuronide

Reactions were carried out in open 2 0 ml plastic test tubes (Sarstedt Ltd , Wexford, Ireland) at 37 °C The reaction was initiated by the addition of the protein solution A sample was immediately removed and analysed by CE It was not necessary to add any material to stop the reaction as the application of the 30 kV separates the components of the reaction mixture thus stopping the opportunity for further reaction Samples were removed approximately every 12 minutes and analysed immediately The reaction was repeated 4 times Control studies were carried out in the absence of 7-hydroxycoumarin, UDP-glucuronic acid and enzyme, respectively

Preparation of standard curve

0-1000 µg/ml standards of 7-hydroxycoumarin-glucuronide were prepared in water The standards were prepared by spiking 10 µl of standard into 40 µl of denatured enzyme preparation, and 50 µl of 50 mM tris-HCl, pH 7 4 It was necessary to denature the protein solution to remove any interference of endogenous β-glucuronidase present If undenatured enzyme preparation was used to prepare the

standards it was possible to monitor the deconjugation reaction of 7-hydroxycoumarin-glucuronide to 7-hydroxycoumarin This was achieved by boiling the solution for 5 minutes and utilising the supernatant after centrifuging the protein solution at 13,000 rpm for 10 min Concentrations of 7-hydroxycoumarin-glucuronide produced were determined from a mean standard curve (n=3) of concentration of 7-hydroxycoumarin-glucuronide versus peak area

2.3.3 CE separation parameters

All capillaries were conditioned each day by 10 min rinses with 0 1 M hydrochloric acid, followed by 0 1 M sodium hydroxide, then deionised water and finally electrolyte buffer

2 3 3 1 Determination of free and total 7-hydroxycoumarin in urine and serum

The capillary used was a 27 cm x 50 µm fused silica column, with a capillary to detector distance of 19 3 cm The capillary was rinsed between runs with 0 1 M NaOH (1 min), followed by 25 mM phosphate buffer, pH 7 5 (1 min) The sample was applied to the capillary by a 3 second pressurised injection (0 5 p s i) and separation achieved with an applied voltage of 20 kV (rise time 0 2 min) at 25 °C Typical running current was 100 µamps The samples were analysed on a Beckman capillary electrophoresis P/ACE 2000 instrument and controlled with System Gold™ software

2 3 3 2 The direct determination of 7-hydroxycoumarin-glucuronide and 7-hydroxycoumarin in urine by capillary electrophoresis

Separations were carried out in a fused untreated silica capillary, 47 cm x 50 µm I D (Beckman Instruments, Fullerton, California, USA) with a capillary to detector distance of 39 3 cm The samples were analysed on a Beckman capillary electrophoresis P/ACE 5500 instrument and controlled with System Gold™ software Separation was optimised under the following conditions The capillary was reconditioned between each run with a 1 5 min 0 1 M NaOH wash, followed by a 3 min rinse with electrolyte buffer The electrolyte solution, for the separation of 7-

hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine, was a 90 10 v/v [100 mM phosphate buffer (pH 7 0) 11 mM deoxycholic acid (sodium salt)] [acetonitrile (Labscan, Dublin, Ireland)] Samples were injected at 0 5 p s i for 5 sec The separation was carried out at 20 kV (rise time 0 2 min) and the typical running current was 160 µamps Detection of the analytes was carried out at 320 nm with a fixed wavelength detector Calibration curves were prepared for each metabolite by plotting the concentration versus peak area Inter-assay and intra-assay variations were assessed to determine the accuracy and precision of the technique

2 3 3 3 The study of coumarin metabolism by human liver microsomes using CE

The capillary used was a 27 cm x 50 µm fused silica column, with a capillary to detector distance of 19 3 cm The preparation step for priming of the capillary was a 1 min rinse with 0 1 M sodium hydroxide, followed by a 1 min rinse with electrolyte solution (25 mM phosphate buffer, pH 7 0 or pH 7 5, depending on the separation required) The sample was applied to the capillary by a 3 second pressurised injection (0 5 p s i) and separation achieved with an applied voltage of 20 kV (rise time 0 2 min) at 25 °C Typical running current was 100 µamps The separation was carried out on a Beckman Capillary Electrophoresis Instrument (P/ACE System 2000) The resultant separation was monitored at 210 nm with a fixed wavelength detector using Beckman System Gold™ software

2 3 3 4 Interspecies differences in coumarin metabolism in liver microsomes examined by CE

The separation was carried out on a Beckman Capillary Electrophoresis Instrument (P/ACE System 5500) The capillary used was a 27 cm x 50 µm fused silica capillary with a capillary to detector distance of 19 3 cm The capillary was purged for 0 75 min with 0 1 M sodium hydroxide Conditioning of the capillary was then achieved by a 1 min rinse with the 50 mM phosphate buffer electrolyte solution (pH 6 8) The sample was applied by a 3 second pressurised injection (0 5 p s i) and the separation was

carried out at 15 kV (rise time 0 2 min) at 25 °C with UV detection at 214 nm 50 mM phosphate buffer (pH 6 8) was found to be optimal, with a working current of 80 μamps

2 3 3 5 The use of CE for studying the in vitro glucuronidation of 7-hydroxycoumarin.

Separation was carried out on a Beckman capillary electrophoresis instrument (P/ACE System 5500) The capillary used was a 57 cm X 50 μm i d untreated silica capillary with a capillary-to-detector distance of 50 cm Between each run the capillary was rinsed for 0 8 minutes with 0 1 M NaOH, then a 2 5 minute rinse with electrolyte solution Samples were applied by 10 second pressurised injections at 0 5 p s i Separation was achieved at 30 kV (rise time 0 2 min) at 25 °C with detection at 320nm Typical running current was 170 μAmps The same capillary was used without any deleterious effects on performance or reproducibility during the duration of the study

2.4 PREPARATION OF LIVER ENZYME HOMOGENATES AND PROTEIN ASSAY

In order to prevent the introduction of contaminating species during the enzyme preparation, all glassware and solutions used for the preparations were autoclaved before use All solutions were prepared in deionised water Human liver microsomes were kindly donated by Dr Uwe Fuhr, University Hospital, Frankfurt am Main, Germany A kit consisting of microsomes of different species (Beagle dog, Cynomolgus monkey, male New Zealand white rabbit, female rat SD-OFA) was purchased from IFFA Credo (L'Arbresle Cedex, France) Protein concentrations of the microsomal preparations were included for each species Livers from other species (gerbil, mouse [Schofield, CD1], bovine, porcine) were obtained from freshly sacrificed animals and stored at -80 °C until required

2.4 1 Preparation of liver microsomes

I *Fragmentation of the sample*

Approximately 1 g of frozen liver was cut into small pieces and washed with 0 9 % sodium chloride until the supernatant remained clear The pieces were blotted dry and weighed

II *Homogenisation*

A 4 fold amount of 0 25 M sucrose solution was then added to the liver pieces and the mixture was homogenised in a Potter homogeniser with a teflon pestle using 5 strokes at 500 min ^{1}

III *Centrifugation*

The solution was centrifuged at 4,000 rpm (1,900 g) for 10 min and 10,500 rpm (13,000 g) for 20 min to remove the nuclei, the mitochondria, unprocessed cells and extracellular matrix After centrifugation the upper lipid layer was removed and discarded The supernatant (including microsomes and cytosol) was then centrifuged at 50,000 rpm (190,000 g) for 80 min to separate out the micrsomes

IV *Wash and centrifuge*

The supernatant was discarded and the pellet was resuspended in an 8 fold amount of 0 15 M KCl and centrifuged at 50,000 rpm (190,000 g) for 80 min

V *Final preparation of microsomal suspension*

The supernatant was discarded and the pellet was reconstituted into a tris (50 mM)-sucrose (0 25M) solution (pH 7 4) Aliquots of the microsomal suspension were then transferred into 2 ml cryotubes and frozen immediately in liquid nitrogen and stored at -80 °C until required Protein content was determined by the BCA assay

2 4 2 Preparation of UDP-glucuronyl transferase

Bovine liver was kindly donated by Kepak Ltd , Meath, Ireland from a freshly sacrificed cow It was immediately put on ice until it could be frozen It was stored at -80 °C until required The preparation was carried out at 0 °C

Approximately 40 g of frozen bovine liver was weighed out and cut into small pieces (0 5 cm X 1 cm X 0 1 cm approximately) It was washed with excess 0 9 % NaCl to remove blood from the liver This washing solution was discarded The liver was homogenised in 4 X weight of the liver of 0 25 M sucrose solution in a 50 ml centrifuge tube The solution was then centrifuged at 4,000 rpm (1900 g) for 20 min The pellet was discarded and the supernatant was added into Beckman quick-seal centrifuge tubes

The supernatant was centrifuged at 50,000 rpm (190,000 g) for 80 min Following centrifugation the supernatant was discarded and the pellet was homogenised in a 0 1 M tris-HCl, 0 1 mM dithiothreitol, and 0 75 % (w/v) sodium cholate solution The solution was centrifuged at 50,000 rpm (190,000 g) for 80 min The pellet was discarded To the supernatant saturated ammonium sulphate was added slowly with gentle stirring to give a 40 % $(NH_4)_2SO_4$ solution This was mixed for 20 min and then centrifuged at 4,000 rpm (1900 g) for 20 min The pellet was discarded To the supernatant saturated ammonium sulphate was added slowly with gentle stirring to give a 70 % solution and mixed for 20 min The solution was then centrifuged at 4,000 rpm (1900 g) for 20 min

The supernatant was discarded and the pellet was resuspended in 50 mM tris-HCl, pH 7 4 and dialysed at 4 °C against 50 mM, tris-HCl, pH 7 4 After dialysis, the solution was aliquoted into 2 0 ml cryotubes and 'snap-frozen' in liquid nitrogen The crude UDP-glucuronyl transferase preparation was stored at -80 °C until required Protein content was determined by the BCA assay

2 4.3 Bicinchoninic acid (BCA) assay for protein determination

The BCA assay was carried out in a 96 well microtitre plate

1 The working reagent was prepared by combining 50 parts reagent A (base reagent containing, sodium bicarbonate, bicinchoninic acid detection reagent and sodium tartrate in 0 2 N NaOH) with 1 part reagent B (4 % copper sulphate solution)

2 A set of BSA standards from 0 - 2 mg/ml were prepared by diluting stock BSA solution in the same diluent as the samples

3 To each respective well 10 µl of standard or unknown was added

4 200 µl of working reagent was then added to each well, mixed, covered and incubated at 37 °C for 30 min

5 The absorbance was read at 562 nm on a microplate reader (Titretek Twinreader PLUS) Concentrations of proteins were determined from a plot of concentration versus absorbance

2.5. 7-HYDROXYCOUMARIN-PROTEIN CONJUGATES AND PRODUCTION OF AN ANTI-7-HYDROXYCOUMARIN ANTIBODY

2 5 1 Production of 7-hydroxycoumarin drug conjugates·

Preparation of diazonium salt of 7-hydroxycoumarin Figure 2 5 1 1 is a schematic representation of the reaction involved in the production of BSA- and thyroglobulin-7-hydroxycoumarin protein drug conjugates

Stage I· *Synthesis and purification of 3-acetylamino-7-aceto-coumarin*

8 1 g 2,4-dihydroxybenzaldehyde, 4 05 g of glycine, 10 g of sodium acetate, and 40 ml of acetic anhydride were added into a 100 ml round bottomed flask and refluxed for 60 min yielding a very dark brown solution which was allowed to cool to room temperature After cooling the semi-solid substance was broken-up and added to 400 ml of water and placed on ice for 30 min The water was poured off and 50 ml of warm methanol was added yielding a mustard coloured solution This was filtered and dried under vacuum The resultant filtrate was recrystallised from acetic acid (the compound was dissolved in minimal acetic acid, with vigorous stirring over heat and the crystals were allowed to form overnight at 4 °C) The compound formed was

filtered and dried under vacuum at 37 °C for several days to remove any remaining acetic acid The identity of the resultant yellow compound was determined from infrared analysis, TLC (the mobile phase used in the TLC separation was a 90 10 (v/v) diethyl ether petroleum ether) and melting point determination

Stage II *Synthesis and purification of 3-amino-7-hydroxycoumarin*

4 0 g of 3-acetylamino-7-aceto-coumarin was refluxed in 250 ml of 3M hydrochloric acid for 30 min until all the solid present was seen to have reacted (i e until the solution went an orange colour) The pH of the solution was neutralised with solid $NaHCO_3$ The resultant yellow compound was filtered under vacuum The compound was dissolved in minimal boiling water then an equivalent amount of methanol was added and the solution was put on ice The resultant recrystallised orange-yellow compound was filtered and its purity and identity was determined by thin layer chromatography (TLC), infra-red analysis (IR) and nuclear magnetic resonance (NMR)

Stage III *Synthesis of a 7-hydroxycoumarin diazonium-ion and its subsequent conjugation to a protein*

To 1 g of 3-amino-7-hydroxycoumarin, 2 5 ml of concentrated HCl was added giving a light brown 'putty' like substance After mixing for 5 min, 2 0 ml of distilled water was added To this 5 ml of 10 % (w/v) $NaNO_2$, at 0 °C, was added dropwise to produce the 3-diazonium ion-7-hydroxycoumarin, yielding an orange compound with a brown crust This was allowed to mix for 20 min at 0 °C To this a 5 % (w/v) protein solution was added dropwise and stirred gently for 5 hr at 0 °C

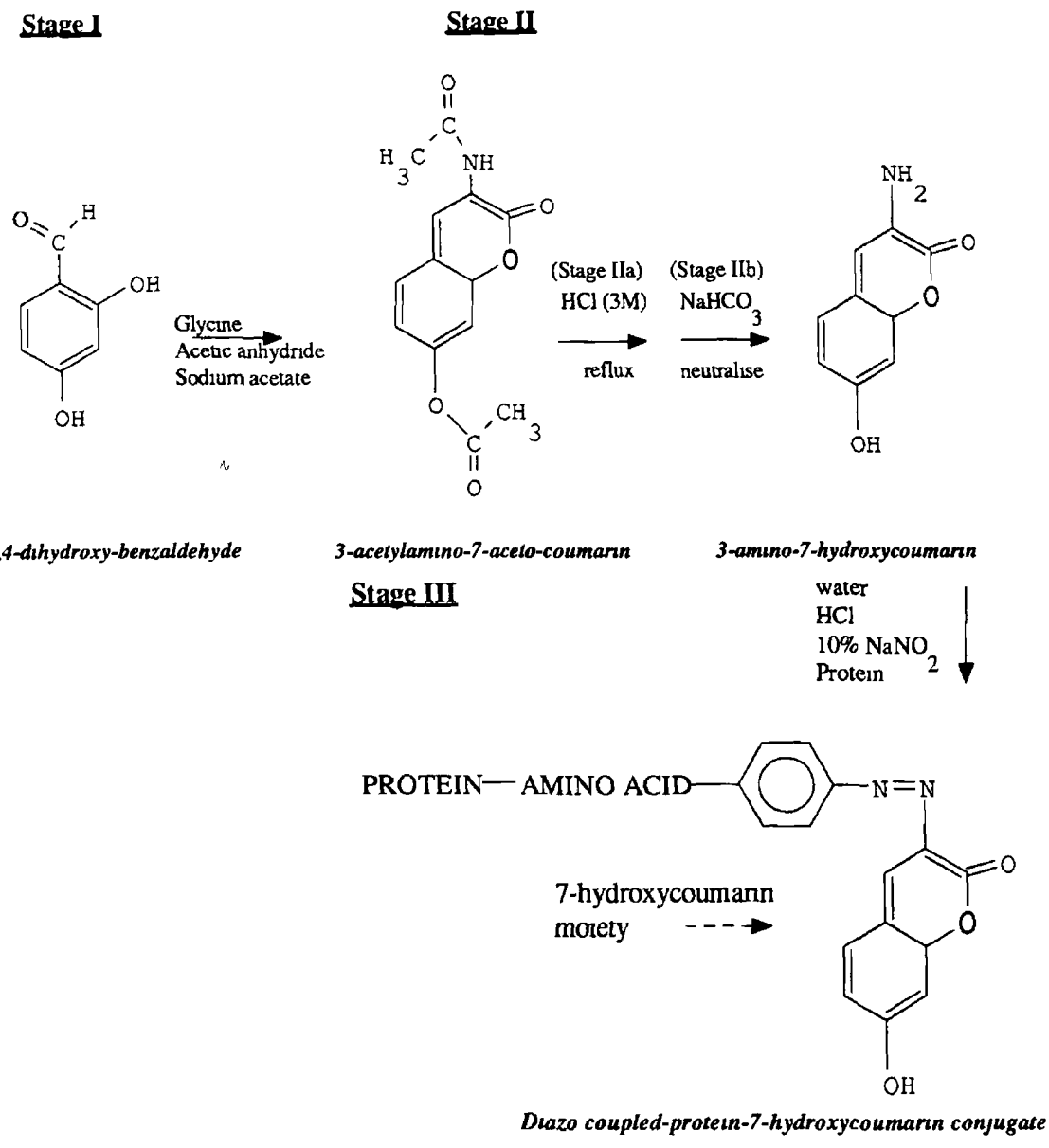


Figure 2 5 1 1 The reactions involved in the production of 7-hydroxycoumarin protein drug conjugates The diagram shows the various stages involved in the preparation of diazocoupled-BSA-or-thyroglobulin-7-hydroxycoumarin protein-drug conjugate

The pH of the reaction was monitored continuously and adjusted when necessary to pH 7 9 with 1 M NaOH The protein-drug conjugates prepared were a bovine serum albumin (BSA) conjugate and a thyroglobulin conjugate The unconjugated 7-hydroxycoumarin was removed by exhaustive dialysis against several changes of distilled water at 4 °C for 48 hr or until the dialysis water remained clear The

coloured solution in the dialysis tubing was reduced by reverse osmosis on a bed of sucrose The remaining solution was removed and freeze dried Conjugation with BSA yielded a rust coloured compound and conjugation with thyroglobulin yielded a brown compound The identity of the conjugate was determined by UV detection, and HPLC with photodiode array detection (see section 5 2 1 3)

Preparation of control protein-conjugate

The above reaction (Stage III) was repeated except for the addition of 3-amino-7-hydroxycoumarin The conjugates produced were compared to the controls produced It was not possible to determine the amount of 7-hydroxycoumarin diazo-coupled to the protein

The thyroglobulin-7-hydroxycoumarin conjugate was used for immunisation and the BSA-7-hydroxycoumarin was used in the screening of the serum for antibodies against 7-hydroxycoumarin

2 5.2. Immunisation protocol:

Licensing

The immunisation procedures were carried out under the supervision, and performed with the appropriate license, of the Department of Health, Dublin, Ireland All appropriate efforts were made to minimise distress and discomfort to the animals used

Initial immunisation

1 ml of a 2 mg/ml solution of thyroglobulin-7-hydroxycoumarin conjugate (prepared in water) was added to 1 ml of Freund's complete adjuvant This was vortexed mixed for 20 sec 1 ml was administered subcutaneously (SC) to each of two New Zealand white rabbits Blood samples were taken pre-injection as control The blood was allowed to clot, and the serum was removed after centrifugation (3,000 rpm for 20 min) The serum was stored at -20 °C until required

Subsequent immunisation (SC)

The procedure was exactly as above except Freund's complete adjuvant (0 02 % w/v) was replaced with Freund's incomplete adjuvant (0 02 % w/v) Booster injections were given every 28 days and blood was obtained 14 days later

2.5 3 Purification of antibody

Ammonium sulphate precipitation

To 30 ml of serum, 30 ml of saturated ammonium sulphate was added dropwise and stirred gently for 60 min on ice at 0 °C The solution was then centrifuged at 4,000 rpm for 30 min The supernatant was discarded and the pellet was reconstituted in 4 ml of ultra-pure water 4 ml of saturated ammonium sulphate was added and stirred as above for 60 min and centrifuged at 4,000 rpm for 30 min The resulting pellet was reconstituted into 0 75 M ammonium sulphate before column purification

Affi-T column preparation

The Affi-T was stored at 4 °C in 0 02 % sodium azide until required It is a thiophilic agent that binds the thiol- groups present in IgG The Affi-T was filtered through a sintered glass filter and washed with ultra-pure water, allowed to dry and then it was added to mobile phase (0 75 M $(NH_4)_2SO_4$ - ammonium sulphate) and poured to form a 9 cm X 1 cm column This was then washed with x10 volumes of mobile phase before the application of the reconstituted pellet The column was connected to a peristaltic pump and the flow rate was adjusted to 1 ml/min The eluent was collected in a fraction collector and high protein content was determined at 280 nm by ultraviolet spectrophotometry

The reconstituted pellet, after the ammonium sulphate precipitation, was applied to the top of the column and allowed to dissipate into the column before the replacement of the mobile phase The IgG present in the solution will interact with the thiophilic agent and will be retained on the column and all other non-specific proteins present will elute in the mobile phase The absorbance was monitored until it decreased below 0 05 O D In order to elute off the antibody it was necessary to switch buffers from the washing buffer to the elution buffer The elution buffer was 50 mM Tris-HCl, pH 7 4

Fractions were analysed as above and those displaying high absorbances were pooled The column was reconditioned by washing through x10 volumes of mobile phase Reverse osmosis of the pooled fractions from several column purifications were carried out on a bed of sucrose solution The resultant solution was dialysed against 0 1 M PBS, pH 7 4, at 4 °C, and stored at -20 °C until required or in 0 02 % sodium azide (w/v) at 4 °C Protein concentration was determined by the bicinchoninic acid assay (see section 2 4 3) and antibody purity was determined by HPLC

2 5 4 Enzyme-linked immunosorbent assay (ELISA).

Screening for antibody production and the determination of 7-hydroxycoumarin by ELISA The ELISA method is based on that of Egan, (1993) and Reinartz *et al*, (1996) Figure 5 1 4 2 1 shows a diagrammatic representation of the ELISA detailed below

I Coating

A 1 mg/ml solution of bovine serum albumin (BSA) BSA-7-hydroxycoumarin (7-OHC) was prepared in 0 14 M phosphate buffered saline (PBS), pH 7 4 A 40 µg/ml solution was the prepared by diluting the stock solution in 0 14 M PBS, pH 7 4 To each well of a 96 well NUNC maxisorb microtitre plate 100 µl was added and allowed to incubate at 4 °C overnight or for 1 5 hr at 37 °C

II Blocking

The plate was then washed 3 times with 0 14 M PBS, pH 7 4 The plate was inverted, shaken and blotted dry A 1 % solution of BSA was prepared in 0 14 M PBS, pH 7 4, and 200 µl was added to each well and incubated at 37 °C for 60 min The plate was then washed 5 times with 0 14 M PBS, pH 7 4, containing 0 05 % (v/v) Tween-20, ph 7 4 and once with 0 14 M PBS, pH 7 4, or the plate was stored in 200 µl of 0 14 M PBS, pH 7 4, containing 0 05 % (v/v) Tween-20, pH 7 4, at 4 °C

III Sample preparation and antibody titre determination

Preparation of 7-hydroxycoumarin standards and samples in urine and their analysis by an enzyme-linked immunosorbent assay (ELISA)

A range of concentrations of 7-hydroxycoumarin (0 - 500 µg/ml) were prepared in 0 14 M PBS, pH 7 4, from a 1 mg/ml stock solution prepared in ethanol 0 14 M PBS, pH 7 4 (20 80 v/v) Into a 10 ml glass tube 0 9 ml of control urine and 0 1 ml of standard was added and vortex mixed Into a 10 ml glass tube, 1 ml of urine sample from a volunteer who had been treated with 250 mg of coumarin, was also added Where necessary the urine sample was diluted with control urine to ensure that the drug level would be within the linear range of the ELISA For the determination of total 7-hydroxycoumarin 1 ml of β-glucuronidase (5000 units/ml, prepared in 0 5 M acetate buffer, pH 5 0) was added to standards and unknown urine samples and incubated for 30 min at 37 °C The samples were then extracted by the addition of 3 5 ml of diethyl ether into the glass tube The tubes were mixed by inversion for 10 min, and centrifuged at 3,000 rpm for 10 min 1 8 ml of the diethyl ether layer was then removed into a 12 mm X 75 mm borosilicate tube and evaporated to dryness The sample was reconstituted into 200 µl of 50 mM phosphate buffer, pH 7 4, immediately before it was required The samples were then diluted 1/10 in 0 07 M PBS, 0 05 % (v/v) Tween-20, pH 7 4, before their analysis The remaining reconstituted sample was used for analysis by CE (see section 2 3 3 1) or HPLC (see section 2 2 3 2)

To each of three wells of a washed plate, 50 µl of the diluted reconstituted samples or standards was added Having determined the optimum dilution (the optimum dilution of the purified antibody prepared for the ELISA was found to be 1/500) of purified antibody (anti-7-hydroxycoumarin antibody) from titre, 50 µl of this dilution [prepared in 1 % (w/v) BSA in 0 14 M PBS, pH 7 4, containing 0 05 % (v/v) Tween-20] was added into each well, mixed gently, and incubated at 37 °C for 2 hr

Determining antibody titre

A range of dilutions of serum, or purified anti-7-hydroxycoumarin antibody solution was prepared in 1 % (w/v) BSA in 0 14 M PBS, pH 7 4, containing 0 05 % (v/v)

Tween-20 The dilutions prepared were from 1/500 - 1/128,000 100 µl of antibody was added to a washed plate and incubated at 37 °C for 1 hr

IV Addition of the 2° Antibody

Horseradish-peroxidase-labelled antibody addition

After incubation the plate was washed 5 times with 0 14 M PBS, pH 7 4, containing 0 05 % (v/v) Tween-20 and then once with 0 14 M PBS, pH 7 4 A working dilution of 1/10000 dilution of the goat anti-rabbit horseradish-peroxidase-labelled antibody was prepared in 1 % (w/v) BSA in 0 14 M PBS, pH 7 4, containing 0 05 % (v/v) Tween-20 100 µl of the goat anti-rabbit HRP-labelled antibody was added to each well and incubated at 37 °C for 1 hr

V Addition of enzyme substrate and colour development

The plate was washed 5 times with 0 14 M PBS, pH 7 4, containing 0 05 % (v/v) Tween-20 and then once with 0 14 M PBS, pH 7 4 The enzyme substrate consisted of 50 mg o-phenylene diamine (OPDA) prepared in 25 ml of 0 15 M citrate-phosphate buffer containing 0 05 % (v/v) Tween-20, pH 5 0 [it was kept in the dark at all times] 5 µl of hydrogen peroxide was added to the substrate just before its addition to each well 100 µl of substrate was added into each well and the plate was incubated for 1 hr at 37 °C The yellow colour was allowed to develop and the absorbance was monitored at 405 nm on a Titretek plate reader

VI Processing of data

The mean absorbances (A) were calculated and divided by the maximal absorbance (A_0) [i e A/A_0] and plotted against the log of the concentration giving a standard curve From this standard curve the concentrations of 7-hydroxycoumarin (total and free) were calculated The antibody titre was calculated from a plot of the log of the reciprocal dilution versus the mean absorbance

CHAPTER 3

CAPILLARY ELECTROPHORESIS AND THE ANALYSIS OF COUMARINS

3.0. INTRODUCTION

Electrophoresis is a technique where, under the influence of an electric field, the migration of a charged particle is monitored. Electrophoresis, in general, is carried out on some form of solid support to facilitate electromigration of those charged particles. Anti-convective media, such as polyacrylamide or agarose gels, are used as the solid support in normal electrophoresis. The classical electrophoresis technique has long analysis times, low efficiency and there are difficulties in detection and automation.

Capillary electrophoresis (CE) is a family of related techniques that employ narrow-bore capillaries, a voltage supply, and selective buffers to perform separations of drugs, proteins, DNA etc. This powerful analytical technique is used for the determination of many species in a multitude of areas and has been reviewed (Wainright, 1990; Li, 1992; Carchon and Eggermont, 1992; Landers, 1993, Landers *et al.*, 1993; Weinberger, 1993; Campos and Simpson, 1993; Marina and Torre, 1994; and Beckman Instruments CE booklets). All major scientific instrumentation companies are now developing separation kits as well as the instrumentation for CE separations. These kits allow the separation of a myriad of compounds including chirals, proteins, and DNA, and facilitate isoelectric point (pI) determination.

The use of capillaries as an electromigration channel presents a unique approach to separation. Capillaries present several advantages over the classical electrophoretic technology. The physical characteristics of narrow-bore capillaries make them ideal for electrophoresis. The large internal surface area make it ideal for efficient heat dissipation. Fused silica capillaries employed in CE typically have internal diameters of 20-100 μm i.d., lengths from 20 cm to 100 cm, and are externally coated with polyimide. This polymeric substance gives excellent flexibility to the capillary and it is easily removed with acid or with a flame to prepare an on-line detection window. The majority of CE is carried out in free solution rather than using an anti-convective medium, although, gels have been used as a sieving media to facilitate some separations.

CE has several advantages over the classical electrophoretic techniques 'Joule heat', caused by the voltages applied for separation, is efficiently removed due to the high surface-to-volume ratio of capillaries Due to this efficient heat dissipation it is possible to increase the voltage supplied and considerably decrease the analysis time, allowing greater resolution Very high electric field strengths (> 500 V/cm) are often used for the separation of many analytes On-column detection and computer controlled systems decreases analysis time Electropherograms often resemble chromatograms thanks to the use of modern detection technology and some form of data output e g computers or an integrator Typical capillaries have total column volumes in the microliter range Minute quantities of electrolyte buffer are thus required to run a CE separation as compared to other liquid chromatographic methods Sample volumes are generally restricted between 1 % and 5 % of total buffer volume i e nanoliter volumes are required for analysis The small volumes will reduce waste as compared to HPLC The majority of buffers used are based on water rather than organic solvents which results in a reduction in organic solvent waste Sensitivities of CE with UV detection are in the order of picomoles (10^{12}) Interfacing of detection systems with laser-induced fluorescence detectors has extended the sensitivity into the attomole (10^{18}) range

The basic instrumental configuration for CE is relatively simple (Figure 3 0 1) All that is required is an untreated fused-silica capillary, a detector viewing window, a controllable high voltage power supply, two electrode assembly, two buffer reservoirs, and an ultraviolet detector The ends of the capillary are placed in the buffer reservoirs and the optical viewing window is aligned with the detector After filling the capillary with electrolyte buffer, the sample can be introduced into the capillary under pressure or electrokinetically

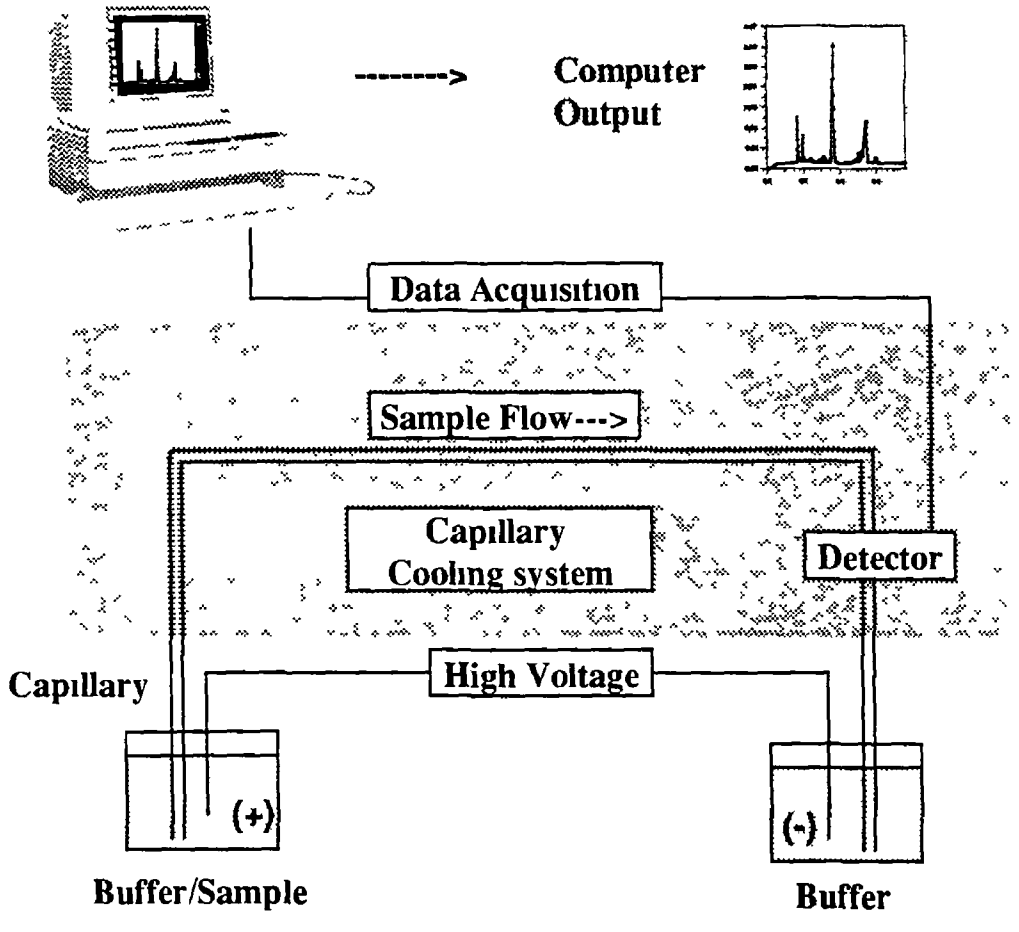


Figure 3 0 1 General schematic of a CE instrument (Diagram taken from Landers et al , 1993) The basic CE instrument consists of a controllable high voltage power supply, two buffer troughs, a cooling system for the capillary, a capillary with on-line UV detector and a means of data acquisition P/ACE System 5500 utilised in the studies described below was a single unit with computer control of all the variables including voltage, detection wavelength, and data acquisition

3 0.1 Capillary Zone Electrophoresis (CZE)

With capillary zone electrophoresis (CZE) the 'normal' polarity is considered to be Inlet (+) → Detector → Outlet (-) as shown in Figure 3 0 1

As electrophoresis ensues under these conditions, the analytes separate according to their individual mobility's and pass the detector as analyte "zones" With 'normal' CE the species migrate as 'positives' → 'neutrals' → 'negatives' and are displayed as an electropherogram The electrophoretic mobility is the principle force in electrophoresis

that carries the analyte zones past the detector window Electrophoretic mobility is the electric force that enables the migration of charged species under the influence of an electrical field This force works in tandem with electroosmotic flow (EOF) Electroosmotic flow is the liquid flow which occurs in the presence of an electric field Without the EOF only positively charged ions would migrate past the detector window However, when the forces work in tandem positive, neutral and negatively charged species can migrate pass the detector window The interaction of an aqueous electrolyte solution with the silica surface in the capillary will cause an excess of negative charge due to the ionisation of the surface's silanol groups The degree of ionisation is controlled mainly by the pH of the electrolyte buffer Electroosmotic flow must be controlled or even suppressed to run certain modes of CE However, the separation of charged species is only made possible by the electroosmotic flow At neutral to alkaline pH, the electroosmotic flow is sufficiently stronger than the electrophoretic migration to allow the migration of all charged species in the CZE separation

Resolution in CE is determined by the following

1 Electroosmotic flow, (dependent on pH or capillary internal surface coating)
2 Electrophoretic mobility
3 Voltage
4 Capillary modifications
5 Electrolyte buffer (dependent on pH, buffer concentrations and constituents)
6 Temperature (determines viscosity)

The pH, ionic strength, organic solvent content, surfactant content, and the presence of chiral selectors will all alter the electroosmotic flow in the capillary A wide variety of buffers can be employed in CE A buffer (Table 3 0 1) is most effective within one or two pH units of its pK_a e g phosphate is used at pH 7 0, and borate at pH 9 0 The choice of concentration is dependent on the separation required, and the dimensions of the capillary used, both of which will in turn determine the voltage applied Buffer additives can be added to alter the separation selectivity The addition of surfactants, cyclodextrins (chiral selectors), organic solvents, cellulose derivatives, urea, and amines

will alter the interactions of analyte with the silanol groups thus improving separation efficiencies Separation of compounds normally unseparable by free solution CE without buffer additives maybe achieved, e g the separation of chiral compounds where under 'normal' CE the isomeric forms would co-migrate

Buffer	Useful pH range
Phosphate	1 14 - 3 14
Acetate	3 76 - 5 76
Phosphate	6 20 - 8 20
Borate	8 14 - 10 14
Zwitterionic Buffers	
MES	5 15 - 7 15
PIPES	5 80 - 7 80
HEPES	6 55 - 8 55
Tricine	7 15 - 9 15
Tris	7 30 - 9 30

Table 3 0 1 Buffers and their buffering regions used in capillary electrophoresis (Table taken from Beckman Instruments, Inc , Introduction to Capillary Electrophoresis)

3 0.2 Capillary Isoelectric Focusing (CIEF)

Carrier ampholytes transport peptides and proteins to their isoelectric points (pI's) A pH gradient is generated when a voltage is passed across the carrier ampholytes present in the capillary The ampholyte mixture separates in the capillary giving a pH gradient under the applied electric field as in typical isoelectric focusing on a solid gel based surface The buffer pH is low at the anode and high at the cathode The positively charged ampholytes will migrate to the negative electrode while the negatively charged migrate to the anode After sample loading the components present will 'focus' at the pH of their respective pI value By changing the anolyte or catholyte solutions, the focused proteins or peptides can be mobilised past the detector to generate an electropherogram If the concentration of protein is too high, it is possible that it will precipitate in the capillary at its pI value as it would on a gel based solid support CIEF

has been applied to peptide mapping, pI determination of proteins and it has been particularly useful for the separation of immunoglobulins, and post-translational modifications of recombinant proteins

3 0.3 Capillary Gel Electrophoresis (CGE)

In capillary gel electrophoresis (CGE) the capillary tubes are filled with a gel which acts as a molecular sieve to produce a size based separation CGE has been useful in the separation of macromolecules such as proteins, DNA polynucleotides, and DNA restriction fragments, because they contain mass-to-charge ratios that do not vary with size Polyacrylamide gel-filled capillaries are usually employed in CGE Agarose gels are unable to withstand the heating produced by the high voltages used in CGE In the separation of proteins the focus has been on the SDS-gels for size based separations The two fundamental classes of gels used are physical and chemical gels The entanglement of polymers in physical gels gives them their porous structure Chemical gels use covalent attachment to form their porous structure

Proteins are generally separated by CGE as follows Proteins are denatured by boiling the sample in 2-mercaptoethanol prior to SDS binding The binding to SDS gives an equal charge-to-mass ratio for all proteins present Separation is then based on size difference Samples are introduced by electrokinetic injection The sample is electrophoresed into the capillary Thus, only charged species are introduced into the system This prevents the extrusion of the gel from the capillary, which would occur if pressurised injection was used Separation times and resolution can be altered by the use of changing the gel concentration, or changing the voltage, providing an adequate heat dissipation system is used

A hydroxypropyl methyl cellulose buffer system and a commercially available coated capillary were found to be ideal for the separation of large oligonucleotides or DNA fragments of 1 kB or larger, in addition to PCR fragment analysis Suppression of the EOF aids in the separation of both DNA samples and proteins This suppression inhibits

interaction and binding of proteins and / or DNA to the capillary wall which may prevent accurate and reproducible separation

3 0 4 Micellar Electrokinetic Capillary Chromatography (MECC)

Micellar electrokinetic capillary chromatography (MECC) [also called micellar electrokinetic chromatography (MEKC)] is a variation on CZE where surfactants (micelles) are added to the electrolyte buffer Surfactants are molecules which exhibit both hydrophobic and hydrophilic characteristics This technique has been investigated in order to obtain selective separation of neutral and ionic compounds while retaining the advantage of CZE Surfactants have polar 'head' groups that can be either cationic, anionic, neutral or zwitterionic and they have nonpolar, hydrocarbon tails (Table 3 0 2)

Type	Name	Formula	CMC[a]
Anionic	Sodium dodecyl sulphate (SDS)	$C_{12}H_{25}OSO_3\ Na^+$	8 1
	Lithium perfluorooctane sulphonate (LiPFOS)	$C_8F_{17}SO_3$	6 72
Cationic	Dodecyl trimethyl ammonium bromide (DTAB)	$C_{12}H_{25}N^+(CH_3)_3Br$	15
Nonionic	Polyoxyethylene (23) dodecanol (Brij-35)	$C_{12}H_{25}(OCH_2CH_2)_{23}O$ H	0 1
Zwitterionic	*N*-dodecylsultaine	$C_{12}H_{25}(CH_3)_2N^+CH2$-$CH_2CH_2SO_3$	1 2
Bile salts	Sodium cholate	$C_{20}H_{33}O_4COO\ Na^+$	12 5
	Sodium deoxycholate	$C_{20}H_{32}O_3COO\ Na^+$	6 4

a CMC values are taken at 25 °C

Table 3 0 2 Some of the surfactants utilised in capillary electrophoresis The table includes their type, name, formulae and critical micelle concentration (CMC) (Table taken from Landers et al , 1993)

If the surfactant concentration exceeds a certain 'critical micellar concentration' (CMC) the surfactant molecules can self-aggregate, forming spherical or helical aggregates called micelles Ionic and hydrophobic sites of interaction are formed when the hydrocarbon tails are oriented toward the centre of the micelle, whilst the polar head group point outward Micellar solutions may solubilise hydrophobic compounds which would otherwise be insoluble in water The aggregation number is the number of surfactant molecules making up a micelle (typically in the range of 50-100) and it and the CMC are both unique to each surfactant Micelles are typically in the range of 3 nm to 6 nm in diameter The separation principle of MECC is based on the differential partition of the solute between the micelle and water whereas CZE is based on the differential electrophoretic mobility

Micellar solutions must be homogenous and not interfere with the detection of the analyte i e they must be UV transparent The use of some surfactants may cause viscous solutions The concentration and physical limitations of these surfactants must be optimised if they are to be utilised for MECC This is also the case for the solubility of the micelle and the ability of the surfactant to form micellar solutions in a particular buffer The hydrophobic and hydrophilic moieties of micelles and binding constants of solutes will determine the micelle mobility When choosing a surfactant (Table 3 0 2) for a particular separation problem it is necessary to first assess the type, structure, size, hydrophobicity, and solubility of the analyte to be separated and analysed The micelle formed by sodium dodecyl sulphate (SDS), an ionic surfactant, migrates toward the positive electrode by electrophoresis However, the EOF transports the bulk solution toward the negative electrode due to the negative charge on the surface of fused silica. The EOF is usually stronger than the electrophoretic migration of the micelle under neutral conditions and, therefore, the micelle is forced to travel toward the negative electrode albeit slower than the EOF which is the basis of MECC separation

When the analyte of interest is added into the micellar solution an equilibrium is formed Some of the analyte is incorporated into the micelle and the remainder is free from the micelle Thus, some of the analyte migrates at the EOF, (that in free solution) and some

will migrate with the micelle The migration velocity, of the analyte, thus depends on the distribution between the micellar and non-micellar (aqueous) phase The analyte will migrate (as a zone) between the migration of the bulk solution and that of the micelle

Another important type of surfactant is the bile salt e g sodium deoxycholate, sodium cholate (Table 3 0 2) They are biological surfactants with a hydrophobic steroid moiety and hydrophilic groups The microenvironment of the micellar bile salts yields different separations in comparison to the other micellar species There is a more polar centre to the micelle as compared to the centres of the long chain alkyl surfactants Another difference is that bile salts form helical micelles, rather than spherical micelles, and they yield selectivities that are significantly different In particular, they are very useful for the separation of very hydrophobic compounds

3 0.5 Conclusion

Other methods for the determination of coumarin and 7-hydroxycoumarin by capillary electrophoresis are described in section 1 2 4 Several CE methods are described below for the separation and determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide after *in vivo* and *in vitro* metabolism Separations are based on capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) CZE was applied to the determination of free and total 7-hydroxycoumarin in urine and serum after deconjugation and extraction of the compound It was also used for the determination of coumarin 7-hydroxylase activity in liver microsomes from various species including man, rabbit, cynomolgous monkey and rat MECC was applied to the direct determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine It was also applied to the determination of 7-hydroxycoumarin-glucuronide after the *in vitro* metabolism of 7-hydroxycoumarin by uridine diphosphate glucuronyl transferase

3 1 CAPILLARY ELECTROPHORESIS AND THE ANALYSIS OF 7-HYDROXYCOUMARIN AND 7-HYDROXYCOUMARIN-GLUCURONIDE

Acknowledgements

The work described below was carried out in conjunction with Professor Malcolm Smyth, and Dr Brian Deasy of the School of Chemical Sciences, Dublin City University, Ireland and Anthony Killard of the School of Biological Sciences, Dublin City University, Ireland The liver samples were obtained from a variety of sources I would like to thank Kepak Meat (County Meath, Ireland) supplies for the bovine liver, and Brian Meehan, School of Biological Sciences, Dublin City University, Ireland, for his help in obtaining the other liver samples and Dr Uwe Fuhr, Department of Clinical Pharmacology, Johann Wolfgang Goethe University, Frankfurt am Main, Germany, for the human liver microsomes

3 1 1 Determination of free and total 7-hydroxycoumarin in urine and serum by capillary electrophoresis

A method has been developed for the determination of 7-hydroxycoumarin in urine and serum, based on deconjugation and extraction of the drug and separation by capillary electrophoresis, with UV detection at 210 nm. Samples were prepared as outlined under section 2 3 2 2 Urine from two volunteers, who had been administered 100 mg of coumarin, was analysed by both capillary electrophoresis and by HPLC The results from the two methods were compared and contrasted

3 1 1 1 Development of the CE separation

Various buffers e g borate, tris-HCl, were investigated in an attempt to separate the compounds Although several of the buffers showed some separation, phosphate buffer was chosen, as it gave rise to the best resolution between coumarin and 7-hydroxycoumarin The pH was then optimised, and the best separation achieved at pH 7 5 The ionic strength of the buffer was also assessed At 0 1 M, a very high running current was exhibited (> 250 μamps), but use of 0 025M phosphate buffer concentration was found to decrease the working current to 100 μamps approximately A CE separation of a mixture of coumarin, 7-

hydroxycoumarin and the internal standard is shown in Figure 3.1.1.1. From this it can be seen that the three compounds are well separated with baseline resolution within 1.2 minutes.

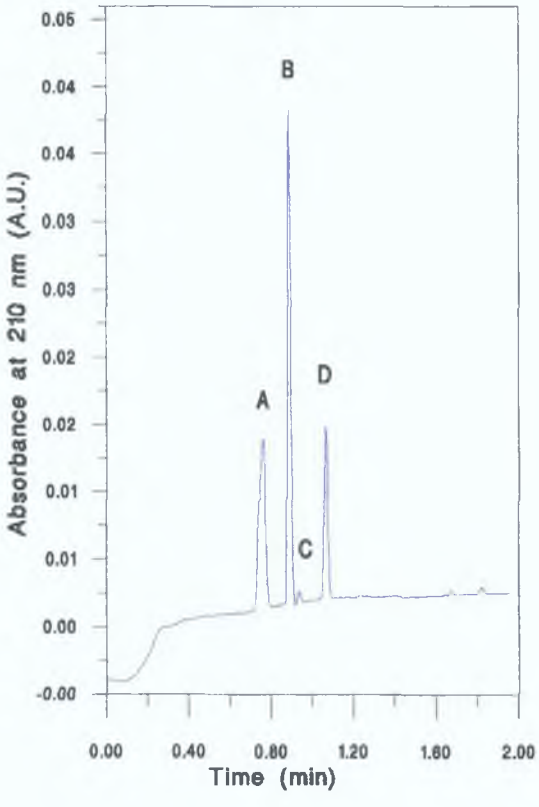


Figure 3.1.1.1. ***Capillary electrophoresis separation of (A) coumarin, (B) 7-hydroxycoumarin, (C) contaminant of 7-hydroxycoumarin, and (D) the internal standard. Samples were prepared in 0.025 M phosphate buffer, pH 7.5. Separation was carried out in a 27 cm untreated silica capillary, using 25 mM phosphate buffer, pH 7.5, with detection at 210 nm.(see section 2.3.3.1.). Each of the peaks shown in the electropherogram is assigned A, B, C or D and this is the protocol throughout each of the figures below.***

Coumarin has a net zero charge at pH 7.5 and it migrates with the solvent front, i.e. at the same migration time as a neutral marker, benzamide. This method cannot be used for the quantification of coumarin in biological fluids due to interferences from endogenous species present in the particular matrix. At the pH utilised one achieves the separation of coumarin from 7-hydroxycoumarin by virtue of the charge difference between the two compounds (7-hydroxycoumarin is more negatively charged at pH 7.5, relative to coumarin). The separation was then applied to the analysis of urine and serum samples after sample

preparation (see section 2.3.2.2.). The separation of the compounds in the urine extract is shown in Figure 3.1.1.2., where it can be seen that there is only minimal interferences to the analysis of 7-hydroxycoumarin from co-extracted endogenous species in the urine.

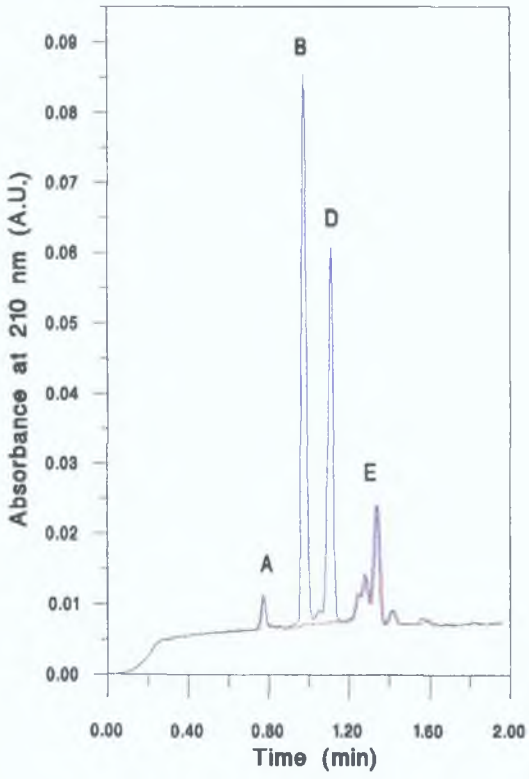


Figure 3.1.1.2. ***Electropherograms of blank urine [red] and 50 μg/ml [blue] 7-hydroxycoumarin standard and internal standard spiked in urine. The samples were prepared as outlined under section 2.3.2.1. Separation was carried out in a 27 cm untreated silica capillary, using 25 mM phosphate buffer, pH 7.5, with detection at 210 nm.(see section 2.3.3.1.). (A) is the solvent front and any neutral compounds, (B) is 7-hydroxycoumarin, (D) is the internal standard, and (E) is co-extracted endogenous species in urine.***

The CE analysis of total 7-hydroxycoumarin in a urine sample taken from a volunteer 6 hours after administration of coumarin is shown in Figure 3.1.1.3. It indicates that most of the interferents have longer migration times compared with those of the drug and its metabolite. This figure also shows that the internal standard cannot be used in the analysis of total 7-hydroxycoumarin owing to the greater number of interferents occurring at its migration time.

There was also irreproducible extraction of the internal standard after the treatment of the urine sample with β-glucuronidase Efforts to find a alternative internal standard which would elute later in the electropherogram and extract reproducibly, proved fruitless The CE analysis of a serum extract is shown in Figure 3 1 1 4 From this it can be seen that there is good separation between the metabolite, the internal standard and the majority of the co-extracted interfering substances There was no interferences from other glucuronides with 7-hydroxycoumarin after treatment of the serum with β-glucuronidase

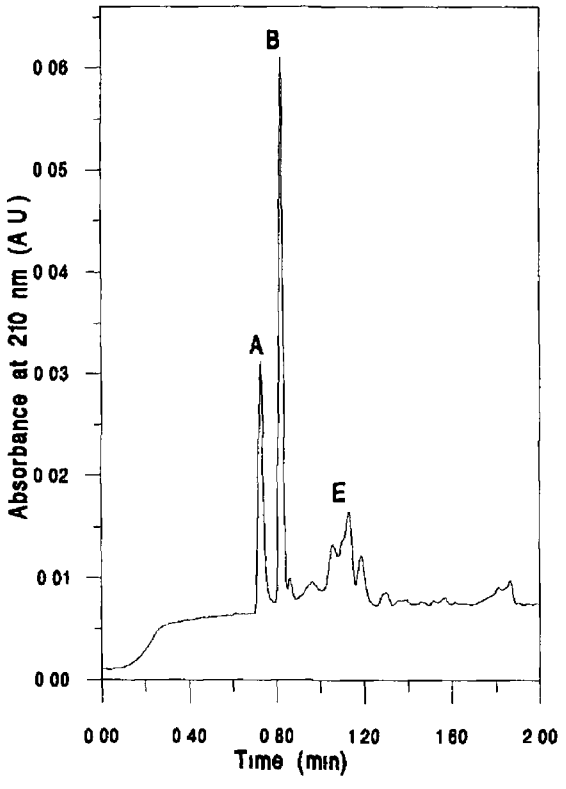


Figure 3 1 1 3 CE analysis of total 7-hydroxycoumarin in a urine sample from volunteer 2, 6 h after administration of coumarin The samples were prepared as outlined under section 2 3 2 1 Separation was carried out in a 27 cm untreated silica capillary, using 25 mM phosphate buffer, pH 7 5, with detection at 210 nm (see section 2 3 3 1) The peaks are (A) solvent front containing any neutral compounds, (B) 7-hydroxycoumarin and (E) co-extracted endogenous species in urine.

The method was then compared to the previously developed HPLC method of Egan and O'Kennedy (1992) for the quantification of 7-hydroxycoumarin in the urine of volunteers who had been administered coumarin When, however, one compares the run times for the samples, one immediately realises the diminution in the time for the determination step (at least eight fold) between the two techniques The method also has a fast regeneration step to recondition the capillary column between every analysis There are no carryover problems, less solvents are used, and there is minimal organic solvent waste as compared with the reversed-phase HPLC method However, as with the HPLC method, a clean-up procedure was used for the biological samples prior to analysis

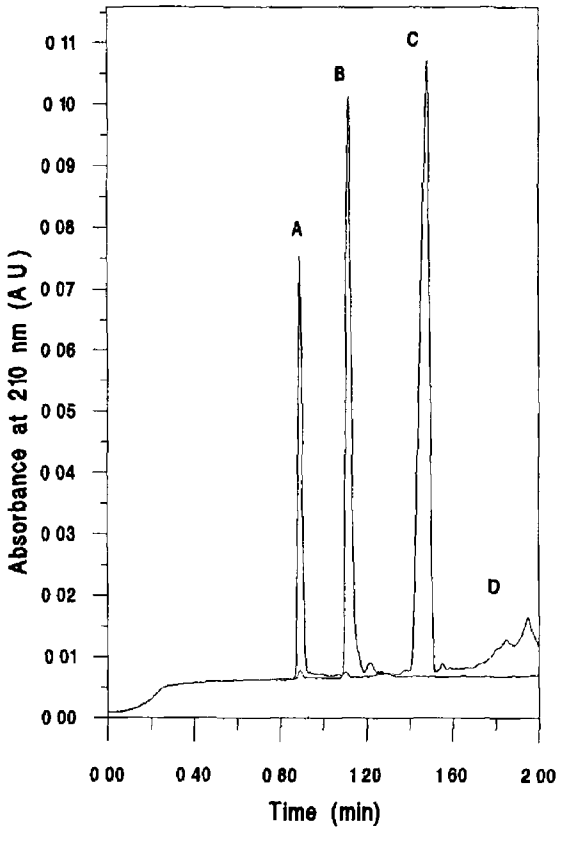


Figure 3 1 1 4 Electropherograms of blank serum [black] and 50 μg/ml [red] 7-hydroxycoumarin standard and internal standard spiked in serum. The samples were prepared as outlined under section 2 3 2 1 Separation was carried out in a 27 cm untreated silica capillary, using 25 mM phosphate buffer, pH 7 5, with detection at 210 nm (see section 2 3 3 1) (A) is the solvent front and any neutral compounds, (B) is 7-hydroxycoumarin, (D) are co-extracted endogenous species in serum.

3 1 1 2 Limit of quantification and linearity

In both urine and serum the limit of quantification of 7-hydroxycoumarin was found to be 1 µg/ml and the linear detection range for the analysis of the drug was from 0 to 50 µg/ml. The mean equation of the line for total 7-hydroxycoumarin is, y = [1 39E-2 ± 6 6E-4] + [1 43E-3 ± 9 3E-5]x and for free 7-hydroxycoumarin is y = [2 16E-2 ± 8 9E-3] + [2 41E ± 2E-3]x, respectively Correlation coefficients were always better than 0 99

3 1 1 3 Accuracy and precision

Tables 3 1 1 1 and 3 1 1 2 are the results from the inter- and intra- assays for the determination of 7-hydroxycoumarin in urine

Concentration Added (µg/ml)	Mean Concentration Calculated (µg/ml)	± Standard Deviation (n=6)	Percentage Relative Standard Deviation (%)
0	0	0	0
1	1 09	0 09	8 9
5	5 16	0 37	7 3
10	10 37	0 58	5 6
20	19 45	0 82	4 2
50	50 85	3 23	6 3

Table 3 1 1 1 Precision and accuracy of the CE method for the analysis of total 7-hydroxycoumarin in urine (Inter-assay) The results were calculated by relating the peak absorbance ratios from each analysis to the mean standard curve to give the mean concentration calculated ± standard deviation and the % relative standard deviation (n=6)

The results are given as the concentration added and the mean concentrations calculated ± standard deviations, and relative standard deviations The inter-assay accuracy and precision for free 7-hydroxycoumarin was determined over a five day period (n=5) The intra-assay accuracy and precision for free drug was determined over five calibration sets on one specific day The inter-assay analysis for total drug was determined over six days (n=6) The use of the internal standard was not of significant benefit for the determination of free 7-hydroxycoumarin

Concentration Added	Mean Concentration Calculated ± Standard Deviation (n=5) (% Relative Standard deviation)	Mean Concentration Calculated ± Standard Deviation (n=5) (% Relative Standard deviation)
(µg/ml)	(µg/ml) (Inter-assay)	(µg/ml) (Intra-assay)
0	0	0
1	0 95 ± 0 07 (7 0)	1 02 ± 0 13 (13 6)
5	5 12 ± 0 26 (5 2)	5 08 ± 0 40 (7 9)
10	9 81 ± 0 21 (2 1)	10 30 ± 0 35 (7 9)
20	21 20 ± 1 00 (4 7)	20 62 ± 0 79 (3 8)
50	51 06 ± 3 84 (7 5)	49 20 ± 2 03 (4 1)

Table 3 1 1 2 Precision and accuracy of the CE method for the analysis of free 7-hydroxycoumarin in urine (Inter [n=5] and intra assay [n=5]) The results were calculated by relating the peak absorbance ratios from each analysis and to the mean standard curve to give the mean concentration calculated ± standard deviation and the % relative standard deviation The results are given as the mean concentration calculated (µg/ml) ± standard deviation about the mean The results in parenthesis are the percentage relative standard deviations (%)

3 1 1 4 Clinical and pharmacokinetic studies

The method developed (see section 2 3 2 1 and 2 3 3 1) was applied to urine samples obtained from two volunteers who had been administered 100 mg of coumarin p o Their urine was analysed for free and total 7-hydroxycoumarin (see Figure 3 1 1 3) During the analysis no coumarin was observed in the volunteer's urine (this was determined from HPLC analysis) Coumarin excretion was calculated by determining 7-hydroxycoumarin in either of the 7-hydroxylated forms (i.e free or glucuronidated) The concentrations of free and total 7-hydroxycoumarin present were calculated from standard curves prepared on the day of analysis and the results obtained were related to the urinary volumes excreted The total amount of 7-hydroxycoumarin excreted in milligrams was related to the amount of coumarin administered The percentage of administered coumarin, excreted as 7-hydroxycoumarin was calculated from molecular weight ratios (Table 3 1 1 3) The results showed that up to 70 % of the coumarin is excreted in the first two hours and that approximately 90 % is excreted within 24 hours These % coumarin excreted correlate with the results of Rautio *et al* (1992) and Egan and O'Kennedy (1992) (see section 3 2 1.) In the comparison of the

results from CE and HPLC analysis of the volunteer's urine samples there was no statistical difference

7-Hydroxycoumarin excreted (mg)

Time (h)	7-OHC excreted CE method Volunteer 1 (mg)	7-OHC excreted HPLC method Volunteer 1 (mg)	7-OHC excreted CE method Volunteer 2 (mg)	7-OHC excreted HPLC method Volunteer 2 (mg)
0	0	0	0 50	6 0
2	64 35	71 85	73 50	75 90
6	23 16	21 99	25 62	21 36
10	3 61	3 30	2 09	2 20
14	1 13	0 93	0	0
24	0	0 30	0	0 40
Total	92 85	98 37	101 71	100 46
% Coumarin excreted*	83 4%	88 7%	92 0%	90 5%

Table 3 1 1 3 Comparison of HPLC and CE analysis of 7-hydroxycoumarin excreted over 24 hours, and percentage of administered coumarin excreted after 24 hours Results are given as the total 7-hydroxycoumarin content in the urine at each time and the total after 24 h The results were related to 100 mg of coumarin administered and expressed as the % coumarin administered that was excreted as 7-hydroxycoumarin (free or conjugated) Volunteer 1 and Volunteer 2 were treated with 100 mg of coumarin * % Coumarin excreted was calculated from molecular weight ratios (0 90) i.e (mol. wt coumarin) / (mol wt 7-OHC)

3 1.2 The direct determination of 7-hydroxycoumarin-glucuronide and 7-hydroxycoumarin in urine by capillary electrophoresis

A method has been developed for the direct determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine without sample clean-up Separation was carried out in 90 % 100 mM phosphate buffer, 11 mM deoxycholic acid (sodium salt) 10 % acetonitrile, on a 47 cm uncoated silica capillary, at 20 kV, with detection at 320 nm. The linear detection range, for concentration versus peak area, for the assay, was from 0

to 100 μg/ml for both analytes with a limit of quantitation of 2 μg/ml for 7-hydroxycoumarin and 5 μg/ml for 7-hydroxycoumarin-glucuronide The method was applied to the direct determination of 7-hydroxycoumarin and the glucuronide conjugate in urine from two volunteers administered with 250 mg of coumarin The samples were analysed by the above CE method (section 2 3 2 2) and by high-performance liquid chromatography (see section 2 2 2 2) There was no statistical difference between the results determined by each of the methods Up to 83% of the coumarin administered was excreted as 7-hydroxycoumarin or 7-hydroxycoumarin-glucuronide The majority of the 7-hydroxycoumarin excreted is in the glucuronide form (98 %) with 2 % occurring as free 7-hydroxycoumarin

3 1 2 1 Development of the CE separation

The best electrolyte solution was found to be a phosphate buffer, deoxycholic acid (sodium salt), acetonitrile mixture Separation of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide prepared in water is shown in Figure 3 1 2 1 It is shown that the 7-hydroxycoumarin and the glucuronide are very well resolved with migration times of approximately 5 min and 7 min, respectively Initially, excellent separation was achieved in borate buffer at pH 9 0, but the 7-hydroxycoumarin-glucuronide was found to be unstable at this pH as it decomposed to 7-hydroxycoumarin during the run time No separation was observed in buffers below pH 6 0 Separation of the analytes prepared in phosphate buffer was carried out in 25 mM phosphate buffer but when assessed in the biological fluids of interest it was found not to be applicable It was not possible to get good resolution of the peaks of interest from the endogenous species present with the 25 mM phosphate buffer, pH 7 5 Separation was achieved at pH 7 0 due to the respective charge difference between 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide The presence of several more -OH groups on the glucuronide would cause it to be more negative than the 7-hydroxycoumarin which has only one -OH group and, thus, enabled separation (see Figure 1 2 2 1) The acetonitrile served, primarily, in decreasing the viscosity of the buffer The phosphate buffer deoxycholic acid mixture is very viscous and the addition of the acetonitrile

reduces this viscosity The optimum level of acetonitrile was found to be 10% as lower or higher amounts altered peak shape and separation efficiency

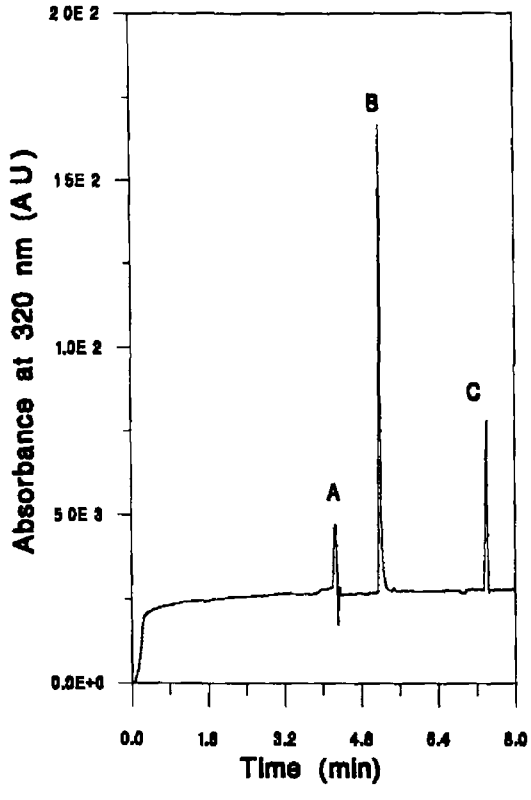


Figure 3 1 2 1 Electropherogram of the separation of (A) water (solvent front) (B) 7-hydroxycoumarin and (C) 7-hydroxycoumarin-glucuronide The sample was prepared as outlined under section 2 3 2 2 Separation was carried out in 90 % 100 mM phosphate buffer, 11 mM deoxycholic acid (sodium salt) 10 % acetonitrile, on a 47 cm uncoated silica capillary, at 20 kV, with detection of the analytes at 320 nm (see section 2 3 3 2)

Acetonitrile is also believed to aid in sample stacking (Shihabi, 1995), and to counteract the deleterious effects of high concentrations of inorganic salts, and to remove proteins present in the sample The removal of proteins and the unwanted ions also prolongs the life of the capillary column

Several micellar systems were tried and it was found that separation was achieved with the use of a bile salt (see section 3 0 4), deoxycholic acid (sodium salt) No separation

was achieved with the anionic surfactant, sodium dodecyl sulphate Deoxycholic acid is also known to complex fatty acids and this may function in a form of on-line sample clean-up The relatively high concentration of phosphate buffer was used to improve peak shape and separation efficiency

It was not possible to monitor for either analytes at 210 nm, as in the above method (see section 3 1 1) as endogenous species in urine were found to co-migrate with the analytes of interest 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide were also found to have an absorbance maxima at 320 nm and in order to increase selectivity, with some loss in sensitivity, this wavelength was chosen as it was found that the interfering species did not absorb at this wavelength Therefore 7-hydroxycoumarin and the glucuronide form could be selectively isolated, resolved, and quantified in urine (Figure 3 1 2 2) It was found that serum had a deleterious effect on the performance of the capillary, and hence it was not possible to analyse for 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in this matrix After trying to analyse one serum sample the capillary would block up and it was then impossible to carry out further analysis with that particular capillary

The analysis of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in plasma samples was also studied but the protein binding of the metabolites, and coumarin, interfered with their accurate determination Plasma samples, obtained from the volunteers who had been administered the 250 mg of coumarin, were analysed Peaks were observed but at longer migration times as compared with spiked samples (results not shown) It was not possible to definitely assign those peaks to 7-hydroxycoumarin or the glucuronide However, there was a significant difference observed on CE between the 0 h, 1 h and 4 h samples, which was also observed with HPLC analysis Different preparation methods of the plasma samples were assessed, with analysis by CE employing the same electrolyte buffer However, treatment of the plasma samples including protein precipitation, addition of organic buffers, sonication and/or heat did not allow the successful determination of the metabolites by CE

The direct determination of any compound without sample clean-up is a very beneficial technique Separations are achieved in less than 7 5 min and the capillary is regenerated in 4 5 min The previous extraction method of Egan and O'Kennedy (1992) involved an incubation step with β-glucuronidase as well as an extraction, evaporation step, reconstitution and, finally, analysis This process involved much labour and was time consuming

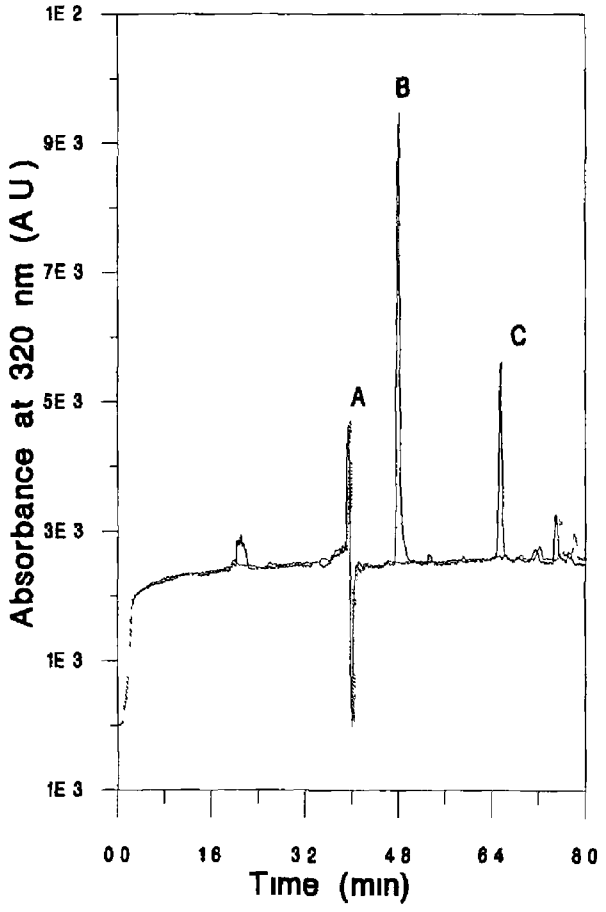


Figure 3 1 2 2 An overlay of the electropherograms, of time (min) versus absorbance (A U) at 320 nm, of the 0 μg/ml [blue] and 100 μg/ml [green] standards prepared in urine and analysed as outlined in section 2 3 2 2 and 2 3 3 2 Separation was carried out in 90 % 100 mM phosphate buffer, 11 mM deoxycholic acid (sodium salt) 10 % acetonitrile, on a 47 cm uncoated silica capillary, at 20 kV, with detection of the analytes at 320 nm. The components are (A) solvent front containing any neutral compounds, (B) 7-hydroxycoumarin, and (C) 7-hydroxycoumarin-glucuronide

No deterioration in capillary performance for the duration of the study was found Several hundred urine samples were passed down the capillary, during the optimisation of the method, and during the inter and the intra assay determinations It is possible to analyse urine and plasma samples directly on HPLC columns but it is not recommended The washing of the capillary, with 0 1 M NaOH, between each run removes any residual components from the urine, thus leaving a new homogenous surface each time

The use of capillary electrophoresis appears, from this study, to be very applicable to the direct determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine However, the method does not allow the determination of coumarin Coumarin acts as a neutral compound at this pH and it migrates with the solvent front and with any other neutral compounds present

3 1 2 2 Limit of quantitation and linearity

7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide, each have two absorbance maxima, at 210 nm and 320 nm The maxima at 320 nm has a lower extinction coefficient than that at 210 nm The limit of quantitation for 7-hydroxycoumarin in urine was found to be 2 µg/ml For 7-hydroxycoumarin-glucuronide it was found to be 5 µg/ml for urine The linear detection range for both analytes was 0-100 µg/ml The mean equation of the line (inter assay) from a plot of the concentration versus the mean peak area are (1) 7-hydroxycoumarin in urine was Y =4 5 E-3 + 4 97 E-3 X, R^2 = 0 99 and for (2) 7-hydroxycoumarin-glucuronide the mean equation in urine was Y = -6 6 E-4 + 2 36 E-3 X, R^2 = 0 99

3 1 2 3 Accuracy and precision

Tables 3 1 2 1 and 3 1 2 2 show the mean concentrations calculated from the intra and inter assays, for the determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine, respectively Percentage relative standard deviations were found to be between 0 5 % and 13 0 % The values were higher at the limit of quantitation of the method The accuracy and precision for the inter-assay was determined over 6 days (n=6) and the intra-assay over 5 (n=5) replicates on one specific day

Concentration Added	Mean Concentration Calculated ± Standard Deviation (n=6) (% Relative Standard deviation)		Mean Concentration Calculated ± Standard Deviation (n=5) (% Relative Standard deviation)	
(µg/ml)	(µg/ml)	Intra-assay	(µg/ml)	Inter-assay
0	0		0	
2	2 86 ± 0 19 (6 8)		1 95 ± 0 25 (13 0)	
5	5 56 ± 0 21 (3 7)		4 97 ± 0 39 (7 9)	
10	9 74 ± 0 19 (1 9)		10 00 ± 0 53 (5 3)	
20	20 16 ± 0 45 (2 2)		20 50 ± 0 70 (3 2)	
50	52 40 ± 4 82 (8 2)		50 49 ± 2 70 (5 3)	
80	78 40 ± 3 40 (4 3)		79 75 ± 0 36 (0 5)	
100	105 25 ± 3 30 (3 2)		97 63 ± 4 48 (4 5)	

Table 3 1 2 1 Precision and accuracy of the CE method for the direct analysis of 7-hydroxycoumarin in urine (Inter [n=5] and intra-assay [n=6]) The results were calculated by relating the peak absorbance ratios from each analysis and to the mean standard curve to give the mean concentration calculated ± standard deviation and the % relative standard deviation The results are given as the mean concentration calculated (µg/ml) ± standard deviation about the mean The results in parenthesis are the percentage relative standard deviations (%)

Concentration Added	Mean Concentration Calculated ± Standard Deviation (n=6) (% Relative Standard deviation)		Mean Concentration Calculated ± Standard Deviation (n=5) (% Relative Standard deviation)	
(µg/ml)	(µg/ml)	Intra-assay	(µg/ml)	Inter-assay
0	0		0	
5	4 53 ± 0 21 (4 6)		4 98 ± 0 62 (12 6)	
10	8 80 ± 0 64 (7 3)		9 80 ± 1 00 (10 2)	
20	20 60 ± 0 56 (2 7)		20 80 ± 1 75 (8 4)	
25	27 70 ± 0 60 (2 1)		25 30 ± 0 96 (3 8)	
50	49 23 ± 3 16 (3 2)		52 90 ± 2 50 (4 8)	
80	77 00 ± 3 23 (4 2)		80 40 ± 4 20 (5 2)	
100	102 25 ± 2 57 (2 5)		100 90 ± 1 70 (1 7)	

Table 3 1 2 2 Precision and accuracy of the CE method for the direct analysis of 7-hydroxycoumarin-glucuronide in urine (Inter [n=5] and intra assay [n=6]) The results were calculated by relating the peak absorbance ratios from each analysis and to the mean standard curve to give the mean concentration calculated ± standard deviation and the % relative standard deviation The results are given as the mean concentration calculated (µg/ml) ± standard deviation about the mean The results in parenthesis are the percentage relative standard deviations (%)

3 1 2 4 Clinical and pharmacokinetic studies

Two healthy volunteers were treated with an oral dose of a 'fast release' 250 mg coumarin tablet Urine samples were taken pre-administration and at specific time intervals after administration of the drug The samples were analysed for the presence of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide by the CE method described in section 2 3 2 2 and analysed as described in section 2 3 3 2 Coumarin content was not determined Figure 3 1 2 3 shows an electropherogram of a volunteers urine sample 10 hours after coumarin administration

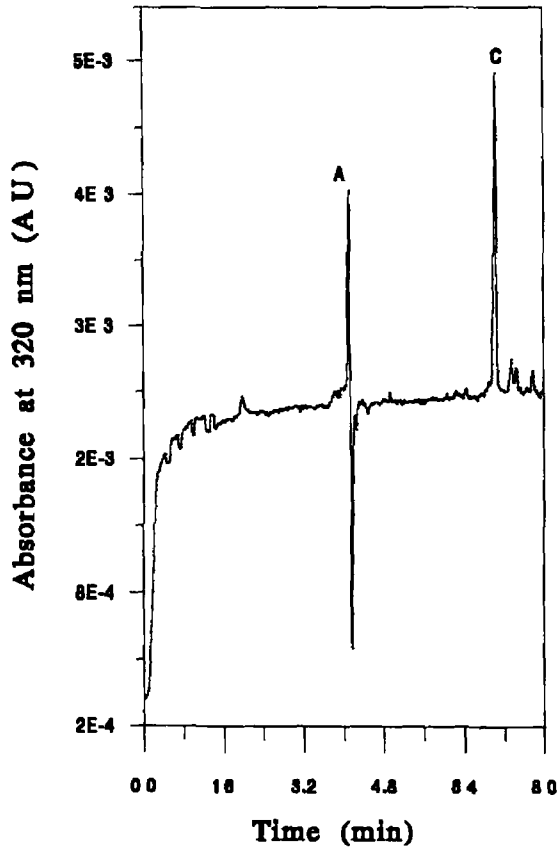


Figure 3 1 2 3 An electropherogram, of time (min) versus absorbance (A U) at 320 nm, of volunteer A's urine sample 10 h after they had been administered coumarin The urine sample was analysed as outlined in section 2 3 2 2 and 2 3 3 2 Separation was carried out in 90 % 100 mM phosphate buffer, 11 mM deoxycholic acid (sodium salt) 10 % acetonitrile, on a 47 cm uncoated silica capillary, at 20 kV, with detection of the analytes at 320 nm The peaks are (A) any neutral compound and the solvent front, and (C) 7-hydroxycoumarin-glucuronide

Slight differences in peak migration times were due to individual differences between samples and standards (i e pH of urine, salts content) Peak identity confirmation was determined by spiking a sample of each standard, 7-hydroxycoumarin or 7-hydoxycoumarin-glucuronide, into the unknown sample and observing the peak increase No other coumarin metabolites were determined The assay developed concentrated on the two major metabolites of coumarin in man Figure 3 1 2 4 shows a plot of the levels of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide excreted by each of the volunteers

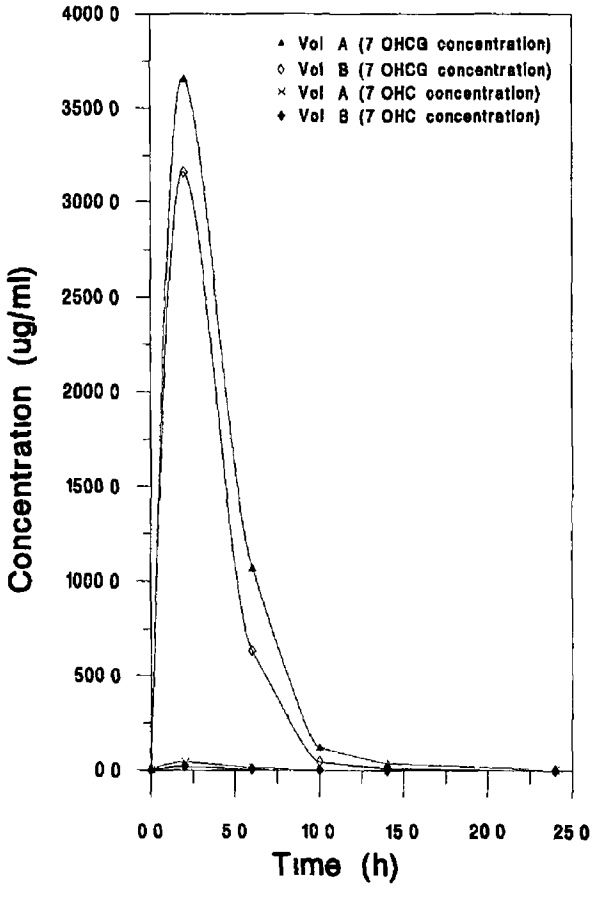


Figure 3 1 2 4 Plot of time (h) versus 7-hydroxycoumarin (7-OHC) and 7-hydroxycoumarin-glucuronide (7-OHCG) concentrations (μg/ml) for each of the volunteers (vol) A and B, determined by CE (method as described in section 2 3 2 2 and 2 3 3 2), after the administration of 250 mg of coumarin Volunteer A's 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide concentrations are shown in blue and red, respectively Volunteer B's 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide concentrations are shown in black and green, respectively

The majority of the 7-hydroxycoumarin excreted is in the glucuronide form (98 %) with 2 % occurring as free 7-hydroxycoumarin in the urine. The urine samples were also analysed by another CE method (see section 2.3.3.1.) and by HPLC (see section 2.2.3.1.). Figure 3.1.2.5. shows an overlay of the results obtained from each of the three methods. The results were compared for the total amount of 7-hydroxycoumarin excreted at the specific timed intervals. Total 7-hydroxycoumarin content was directly determined or calculated from the addition of free and glucuronidated 7-hydroxycoumarin present in the urine samples. The total conjugated 7-hydroxycoumarin was determined from the 7-hydroxycoumarin-glucuronide content multiplied by the molecular mass ratio between 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide (i.e. 162.1/338.1 x 100/1 = 47.9%).

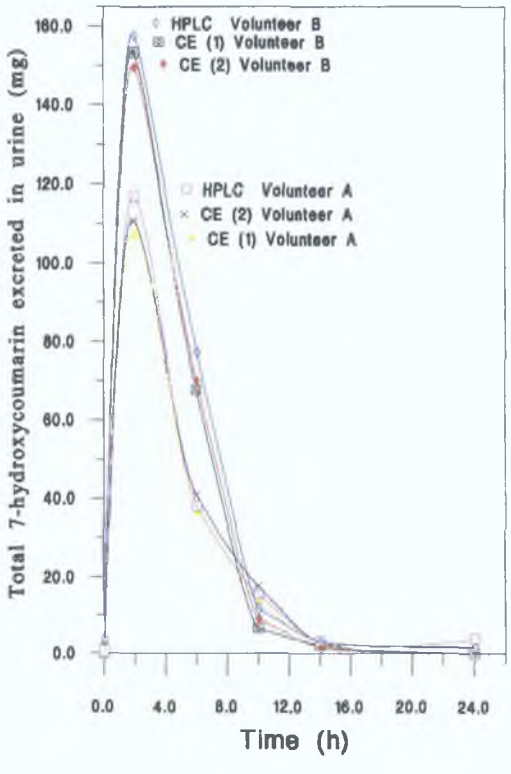


Figure 3.1.2.5. Plot of time (h) versus total 7-hydroxycoumarin excreted (mg) for each of the volunteers A and B, determined by two CE methods, CE (1) method [Volunteer A's and B's profile is shown as the yellow and black, respectively (see section 2.3.3.1.)]; CE (2) method [Volunteer A's and B's profile is shown as the green and red, respectively (see section 2.3.3.2.)]; and by HPLC [Volunteer A's and B's profile is shown as the purple and blue, respectively - see section 2.2.3.1.].

This result was then related to the urinary volume to determine the total amount of 7-hydroxycoumarin excreted 54% of the coumarin administered was excreted in the first two hours as 7-hydroxycoumarin or 7-hydroxycoumarin-glucuronide with up to 83 % being excreted within 24 hours In the comparison of the results from the three methods there were no significant statistical differences (3 0 %) The CE method allowed the direct determination of each of the compounds without the addition of β-glucuronidase and with minimal sample preparation Results were obtained in shorter time than either the CE method (see section 2 3 3 1) or by the HPLC method (see section 2 2 3 1)

Capillary electrophoresis was applied to the determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in biological fluids (i e urine and serum) Each of the two methods developed were found to be both accurate and precise Both methods were applied to the determination of the metabolites in urine samples from two volunteers who had been treated with coumarin The first CE method described was based on a deconjugation, extraction procedure with separation on CE Detection was carried out at 210 nm The availability of pure 7-hydroxycoumarin-glucuronide standard allowed for the development of a method for the determination of 7-hydroxycoumarin-glucuronide after coumarin metabolism

In the second method there was minimal sample preparation, and results from analysis were obtained within 7 5 minutes for each sample The long preparative steps involved in the first method were overcome by the use of the different buffer system and by detecting the analytes at 320 nm rather than 210 nm Although there is a loss in sensitivity, the benefits of quicker preparation times make the second method a more favourable method for the determination of both 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide simultaneously However, the use of CE with UV detection does not allow the determination of very low levels of metabolites

CE is an ideal method for the determination of many of the coumarins especially for metabolite analysis The potential for coumarin analysis is enormous The next section

deals with an *in vitro* procedure for determining the metabolism of coumarin and 7-hydroxycoumarin Thus, CE has been shown to be very effective for determining several coumarin *in vivo* metabolites and in section 3 2 it is shown that CE may be used for determining Phase I and Phase II coumarin metabolism *in vitro*

3 2. CAPILLARY ELECTROPHORESIS AND METABOLISM STUDIES

3 2 1 The metabolism of coumarin (a review)

Coumarin and its derivatives are metabolised in a number of ways, which vary with the substrate, the species, and the presence of other drugs Coumarin has been the basis for intense interest in metabolism studies as it is ideal for determining the activity of a drug metabolising enzyme called Cytochrome P4502A6 (Pelkonen *et al*, 1996) As a relatively simple organic structure it is metabolised to a large variety of products (Figure 3 2 1 1) The clinically observed effects of coumarin, the toxicity or lack of either are generally attributed to coumarins' metabolites However, there has been some suggestions that the highly aromatic nature of coumarin may cause some of the observed side effects in patient trials (Marshall *et al*, 1994-personal communication) Figure 3 2 1 1 shows some of the possible metabolic pathways that coumarin undergoes after administration The possible pathways include hydroxylation in the 3-,4-,5-,6-,7- and 8-positions and metabolites formed by the opening of the ring structure The metabolism of coumarin via 7-hydroxycoumarin to the glucuronide metabolite is given in Figure 1 2 2 1 The metabolism of coumarin via 3-hydroxycoumarin to ring opened products is also shown

In man, the major Phase I metabolite of coumarin is 7-hydroxycoumarin and the main Phase II metabolism of 7-hydroxycoumarin is 7-hydroxycoumarin-glucuronide 7-hydroxylation of coumarin is catalysed by a specific cytochrome P450 In man this is Cytochrome P450 2A6 (CYP2A6)

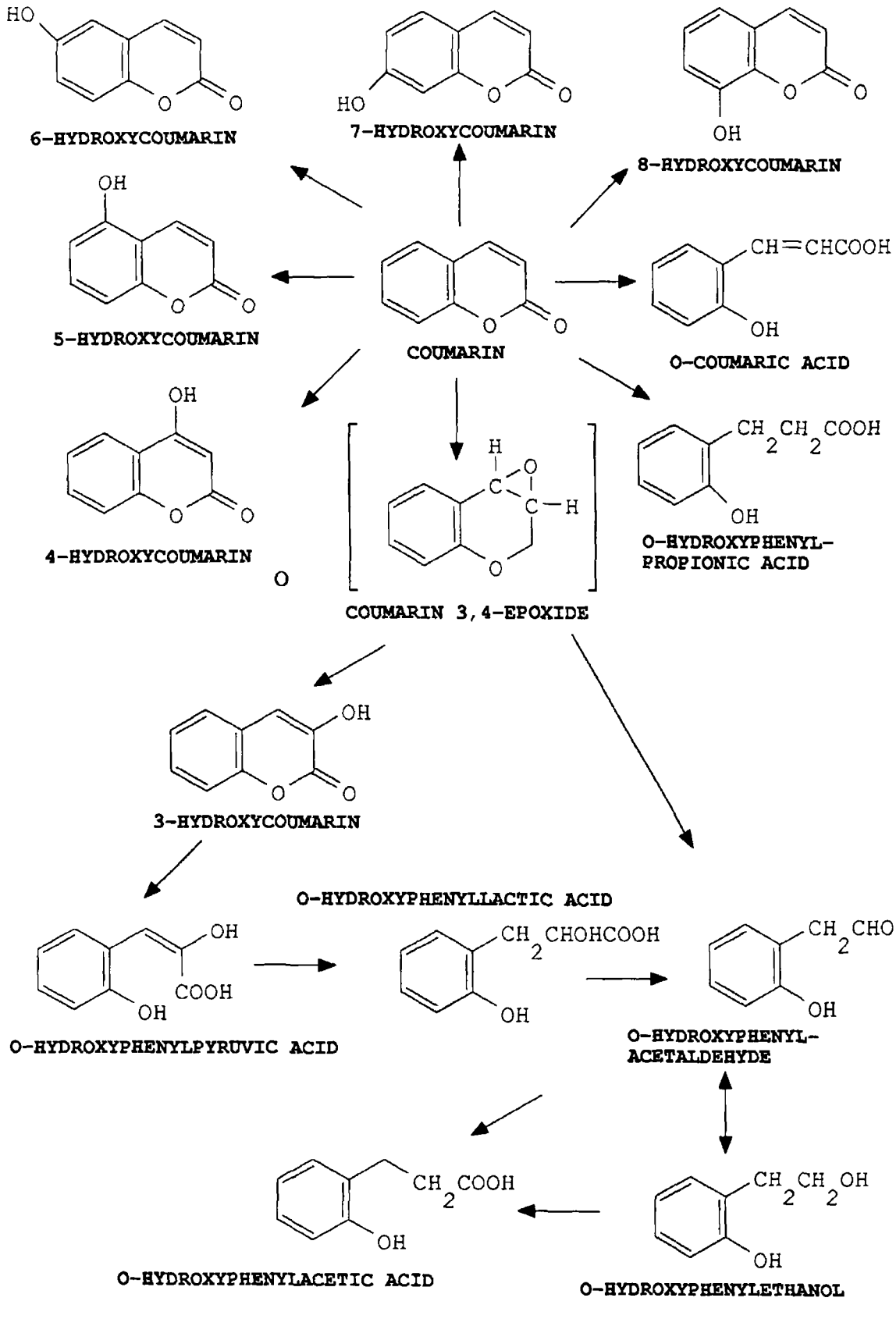


Figure 3 2 1 1 Possible metabolic pathways of coumarin in mammals [Taken from Walters et al (1980), Fentem et al (1991), and Pelkonen et al (1996)]

The analagous isoform of the enzyme in other species are refered to as CYP2A5 in mouse and CYP2A3 in rat In some species, e g rat, 7-hydroxylation is only a minor pathway by which coumarin is metabolised The rat reportedly metabolises coumarin via the 3-hydroxycoumarin pathway The species difference in coumarin hepatotoxicity is believed to be due to the metabolic pathway used and the metabolites formed (Fentem and Fry, 1993)

The majority of work in the area of coumarin metabolism has focused of coumarin 7-hydroxylase activity both *in vivo* and *in vitro* Table 3 2 1 1 is a list of the species and their respective levels of coumarin 7-hydroxylase activity The data in the table relates to the *in vitro* study of coumarin metabolism It is clearly seen that there is a great difference in coumarin 7-hydroxylase activity between species and also within species The species with the highest reported coumarin 7-hydroxylation are human and monkey The Mongolian gerbil also has a high level of enzyme activity Within man there has been a focus on interindividual variability The production of the other metabolites will not be covered in this chapter The emphasis in the section below is on the *in vitro* metabolism of coumarin to 7-hydroxycoumarin and the *in vitro* metabolism of 7-hydroxycoumarin to 7-hydroxycoumarin-glucuronide

Table 3 2 1 2 contains a list of the interindividual differences in coumarin 7-hydroxylation in population studies in man It is clearly seen that there is widescale variability in the coumarin 7-hydroxylase activities in man The data presented in the table is given as a mean ± standard deviation for the patients analysed However, the variation in total 7-hydroxycoumarin excretion was from 17 % - 100 % and 20% - 100 % (Rautio *et al*, 1992, and Iscan *et al*, 1994) The mean, thus, does not give an accurate indication of the ranges of 7-hydroxycoumarin excretion covered However, within the populations there is a similar profile to coumarin 7-hydroxylation The mean is approximately 69 3% ± 10 6 % from the data in the table The methods of 7-hydroxycoumarin determination after coumarin metabolism are outlined in section 1 2 1 3, 1 2 2 1, and 1 3 1

Species	Coumarin 7-hydroxylase activity	Coumarin 7-hydroxylase activity ± Standard Deviation
	(nmol/g liver/min)	(pmol/mg protein/min)
Man	14-47	999 ± 49 (100-5000)
Mouse DBA/2 cntr	6 2	38 ± 13
PB-treated	19 8	
Mouse C57Bl/6 cntr	0 9	
PB-treated	4 3	
Rat (Wistar)	< 0 5	
Rat (Sprague-Dawley)	< 0 5	nd
Guinea Pig	5 8	95 ± 10
Rabbit (New Zealand)	3 1	357 ± 36 (480)
Syrian Hamster		320 ± 60
Chick Embryo		1200
Cynomolgus Monkey		3470 ± 374 (1780)
Calf liver slices		869 ± 133
Crayfish		25 ± 4
Pig		315 ± 31
Cat		10
Dog		200
Rat control		3 ± 1
Phenobarbitone treated		9 ± 1
β-naphthoflavone treated		7 ± 1
Isoniazid		4 ± 2
Pregnenolone 16α-carbonitrile		11 ± 1
Mongolian Gerbil control		682 ± 85
Phenobarbitone treated		1504 ± 92
β-naphthoflavone treated		844 ± 22
Isoniazid		378 ± 10
Pregnenolone 16α-carbonitrile		361 ± 8

Table 3 2 1 1 Table of coumarin 7-hydroxylase activities in liver microsomes and liver homogenates [The data was compiled from- Kaipainen et al, (1985), Fentem and Fry, (1991), Goeger and Anderson, (1992), Pelkonen et al, (1993), (1996), and Lake et al, (1995)] Results in parenthesis are from a different source but for a similar microsomal metabolism study

Coumarin administered	Total 7-OHC excreted as % of coumarin admin.	Reference
5 mg	59 8 % ± 21 5 % (n=100)	Iscan *et al* 1994
5 mg	64 0 % ± 15 0 % (n=110)	Rautio *et al* 1992
200 mg	63 4 % ± 16 6 % (n=7)	Egan *et al* , 1990
2 mg	86 0 % ± 3 0 % (n=64) 79 0 ± 4 0 % (n=64)	[1]Cholerton *et al* , 1992
10 mg	63 7 ± 12 0 % (n=13)	Merkel *et al* , 1994

1 Cholerton et al 's results were from the comparison of two analytical methods

Table 3 2 1 2 List of the interindividual differences in coumarin 7-hydroxylation in population studies in man The results are given as the mean total % of coumarin excreted as 7-hydroxycoumarin ± the standard deviation and 'n' is the number of volunteers involved in the trial

The primary metabolic fate of coumarin is hydroxylation at all six of the possible ring positions (3-, 4-, 5-, 6-, 7-, and 8- hydroxycoumarins) or by opening of the heterocyclic ring Ring opening eventually leads to the production of o-hydroxyphenylpropionic acid, o-hydroxyphenylacetaldehyde, o-hydroxyphenylethanol, and o-hydroxyphenylacetic acid (Figure 3 2 1 1) The production of some of these compounds has been attributed to the production of a reactive intermediate, coumarin 3,4-epoxide (Fentem *et al* , 1991) They reported on the identification of o-hydroxyphenylacetaldehyde as a major novel metabolite of coumarin formed by rat, gerbil and human liver microsomes It was postulated that hepatotoxicity may be due to the conjugation of the coumarin 3,4-epoxide to glutathione or the covalent binding to cellular microsomes Another coumarin metabolite is 6,7-dihydroxycoumarin

3 2 1 1 The in vitro study of coumarin metabolism

Reaction (1) is a representation of a typical reaction scheme where the substrate (SH) may represent a steroid fatty acid, drug or other chemical that has an alkane, alkene, aromatic ring or heterocyclic ring substituent that can serve as a site for oxygenation

Reactions of interest in the incubation solution

P450

(1) $NADPH + H^+ + O_2 + SH$ ----------> $NADP^+ + H_2O + SOH$

NADPH (nicotinamide adenine dinucleotide phosphate) is needed to donate 2 electrons to the heme iron of the cytochrome P450 enzyme In the absence of this co-factor, no reaction will occur SOH in the above reaction represents the oxidised product Reaction (2) is the cofactor reaction (part of the pentose-phosphate pathway) which allows reaction (1) to occur

G-6-P-dehydrogenase

(2) $NADP^+$ + *Glucose-6-phosphate* --------> *NADPH + 6-phosphogluconolactone*

$NADP^+$ is initially converted to NADPH by the use of glucose-6-phosphate-dehydrogenase and this reduced form of the cofactor enters the second reaction where coumarin is hydroxylated to 7-hydroxycoumarin The $NADP^+$ produced in this reaction can now re-enter the first enzyme reaction and can be recycled in this way Reaction 1 is a representation of coumarin metabolism where SH is coumarin (Figure 1 2), and SOH is the hydroxylated form i e 7-hydroxycoumarin (Figure 1 3) These reactions were used in the preparation and study of coumarin 7-hydroxylase activity in microsomes from humans and from various species

Sections 3 2 2 1 and 3 2 2 2 following give the results from two studies carried out on CE for the determination of 7-hydroxycoumarin after coumarin metabolism

3 2.2. Interspecies Differences in Coumarin Metabolism in Liver Microsomes examined by Capillary Electrophoresis

3 2 2 1 The study of coumarin metabolism by human liver microsomes

3 2 2 1 1 Development of the CE separation

The CE separation method developed for the determination of 7-hydroxycoumarin (see section 2 3 3 1) after the *in vivo* metabolism of coumarin was adapted for the rapid analysis of the *in vitro* metabolism of coumarin Liver microsomes were prepared or obtained from various sources and used in determining coumarin 7-hydroxylase activities Capillary electrophoresis was shown to be an excellent technique for the study of coumarin metabolism to 7-hydroxycoumarin *in vitro* The method was applied to the determination of 7-hydroxycoumarin production by human liver microsomes and in a follow-up study by microsomes from various different species The species studied included rabbit (New Zealand white), mouse (CD1, Schofield), rat (wistar), bovine, porcine, cynomolgus monkey, gerbil, and dog

Both studies used the same incubation solution and sample preparation (see section 2 3 2 3) However, there was some variations between the CE methods used for either study (see sections 2 3 3 3 and 2 3 3 4) The preparation of microsomal solutions from a liver sample is described in section 2 4 1 above The CE method developed allowed the investigation of coumarin metabolism to 7-hydroxycoumarin without sample clean-up and results were available within 2 min Coumarin is prepared in a minimal volume of methanol (0 09 % of total volume), which is known to inhibit cytochrome P450 activity Separation was achieved in 0 025 M phosphate buffer, pH 7 0 with detection at 210 nm Figure 3 2 1 2 shows the separation of the incubation mixture at 0 min and 45 min after the reaction commenced When analysed on the CE using 25 mM phosphate buffer, pH 7 0, both $NADP^+$ and NADPH exhibited very poor peak shapes (results not shown) It was not possible to determine peak absorbances or areas due to the poor peak shape exhibited However, when $NADP^+$ and NADPH are analysed on the CE using 25 mM phosphate buffer, at pH 7 5 (Figure 3 2 1 2), it was possible to determine

peak areas and absorbances (see section 3 2 2 1 3) The absorbance of coumarin and 7-hydroxycoumarin could both be monitored with good sensitivity at 210 nm

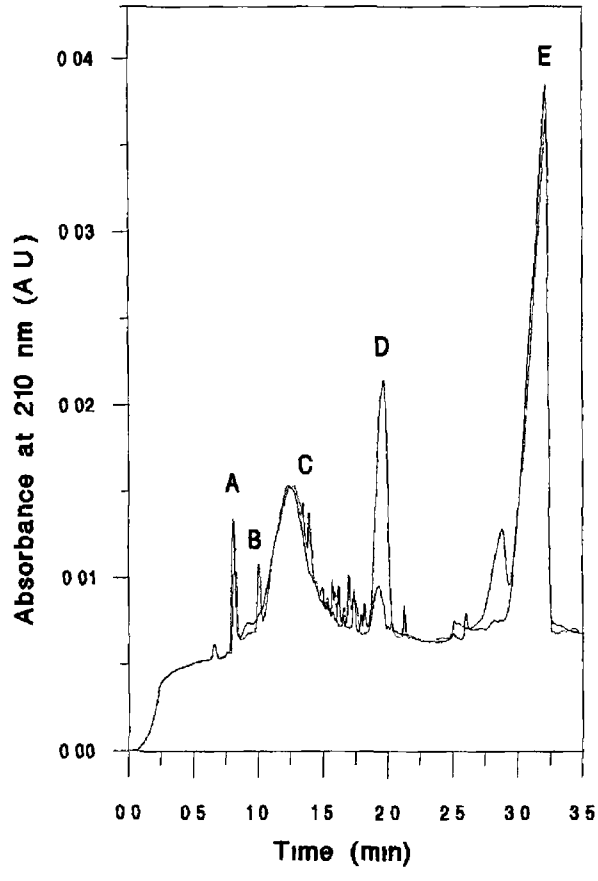


Figure 3 2 1 2 Overlay of electropherograms showing separation of (A) all neutral compounds including coumarin, (B) 7-hydroxycoumarin, (C) microsomes, (D) NADP$^+$ and (E) NADPH The samples were analysed at 0 min (green) and at 45 min (red) Separation was carried out in a 27 cm untreated silica capillary, using 25 mM phosphate buffer, pH 7 5, with detection at 210 nm. The samples were separated as described in section 2 3 2 3 and 2 3 3 3

Figure 3 2 1 3 shows an overlay of the typical electropherograms at 0 min and after 45 min for the metabolism incubation for the P18 microsomal preparation The metabolite, 7-hydroxycoumarin, migrates at 0 9 minutes and its increase can be followed However, it is not well separated from the broad peak of the microsomal preparations at pH 7 5 and, thus, it must be monitored at pH 7 0 (Figure 3 2 1 3) to resolve the compounds It can be seen from Figure 3 2 1 2 that NADP$^+$ and NADPH are well separated and quantifiable at this pH

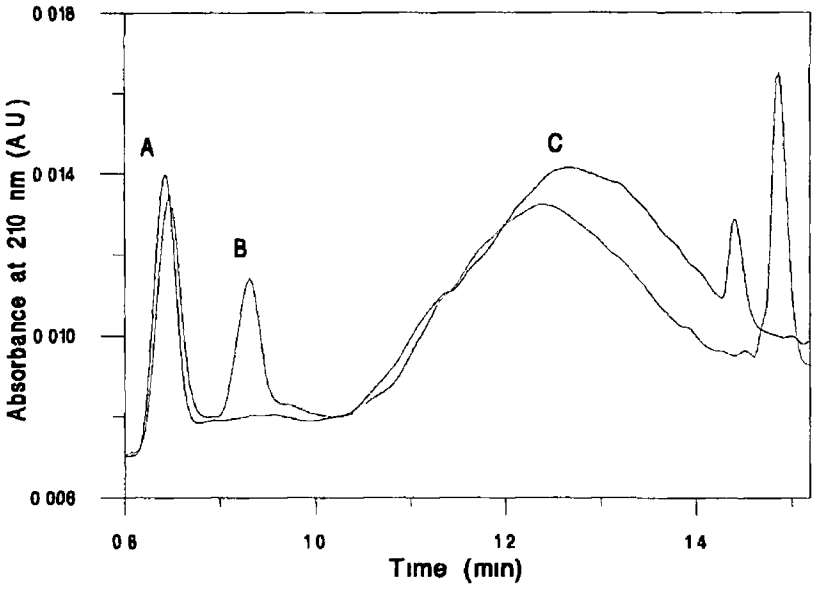


Figure 3 2 1 3 Overlay of electropherograms showing CE separation of (A) all neutral compounds including coumarin, (B) 7-hydroxycoumarin, and (C) microsomes The samples were analysed at 0 min (black) and at 45 min (red) Separation was carried out in a 27 cm untreated silica capillary, using 25 mM phosphate buffer, pH 7 5, with detection at 210 nm The samples were separated as described in section 2 3 2 3 and 2 3 3 3

A neutral marker (benzamide) was run under the separation conditions above (see section 2 3 3 1), and it was found to co-migrate with coumarin, thus suggesting that coumarin is behaving as a neutral molecule at this pH By knowing the migration time of the neutral marker it is possible to say that all the compounds that have longer migration times than coumarin are negatively charged and hence migrate more slowly than coumarin 7-hydroxycoumarin is more negatively charged than coumarin due to the hydroxyl group at the 7-position This hydroxyl group is partially ionised in the phosphate buffer at pH 7 0 The microsomes migrate more slowly than the 7-hydroxycoumarin and are seen as a broad peak due to the different types of proteins in the microsomal suspensions $NADP^+$ has a structure with four negative charges and a

positively charged nitrogen associated with it This suggests an overall charge of 3 negative charges This high overall negative charge explains the slow migration of $NADP^+$ The NADPH molecule has a similar structure to $NADP^+$ but the positive charge on the nitrogen is no longer present (Figure 3 2 1 4)

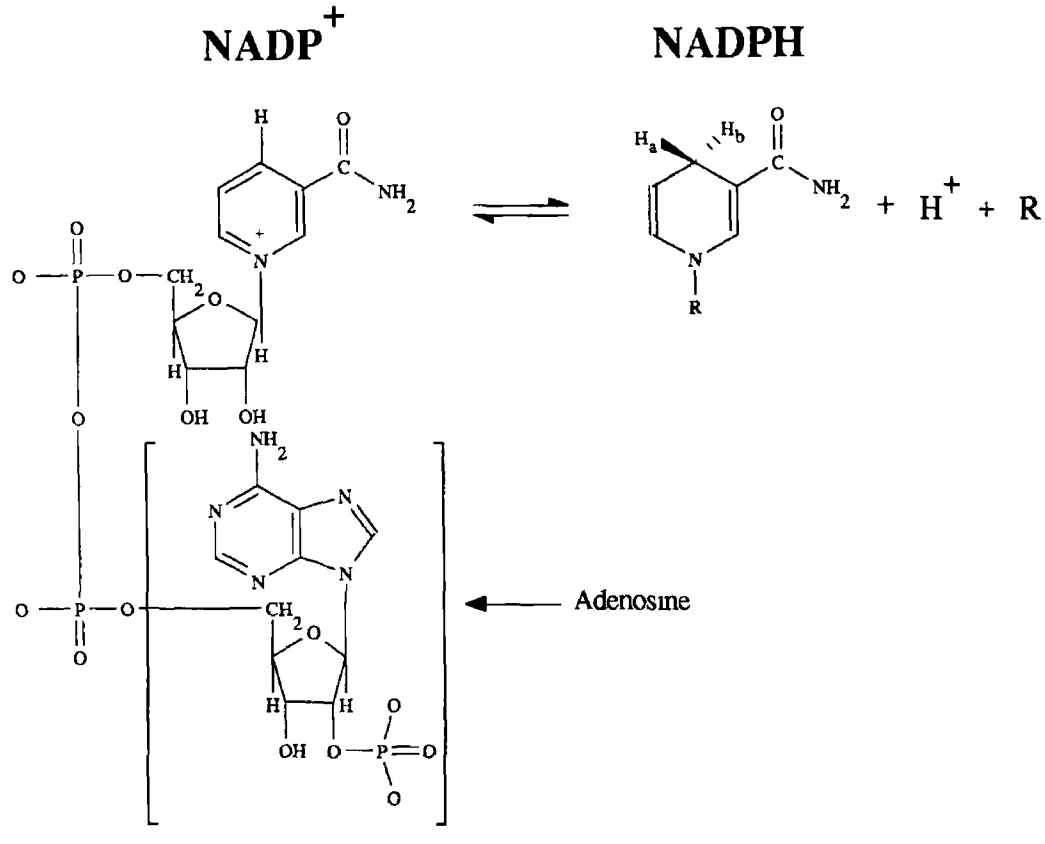


Figure 3 2 1 4 ***Chemical structures of $NADP^+$ and NADPH***

As a result, this more negatively charged compound has the slowest migration rate of all the compounds Control incubations included an absence of (A) coumarin, (B) microsomes, and (C) NADPH-regeneration system Coumarin was not quantifiable under these conditions as other unknown reactions were occurring producing neutral compounds which co-migrate with coumarin The neutral compound produced was dependent on the NADPH reaction as observed with control (A).

3 2 2 1 2 Coumarin metabolism profiles

Microsome preparations from 5 different patients were used in metabolism studies (Table 3 2 1 3) Table 3 2 1 3 gives some clinical details on the sources of liver samples from which the microsomes were prepared Incubations were carried out in triplicate

Liver	Donor sex	Donor age	Smoking status
P13	F	64	N/S
P14	F	48	N/S
P17	F	54	S
P18	F	44	N/S
R1	M	54	N/S

Table 3 2 1 3 Details of liver donors including their age, sex, and smoking status

Figure 3 2 1 5 shows a plot of concentration of 7-hydroxycoumarin [(nmol / mg protein) ± Std Deviation], versus time for each of the five metabolic studies There was no significant difference observed between the coumarin 7-hydroxylase activity for the smoker as compared to the non-smokers It shows that each of the microsomal preparations displays differing coumarin metabolism profiles Although the standard deviations about the mean concentrations calculated are relatively high, this corresponds to microsomal drug metabolism results from other researchers (Fuhr *et al* , 1990)

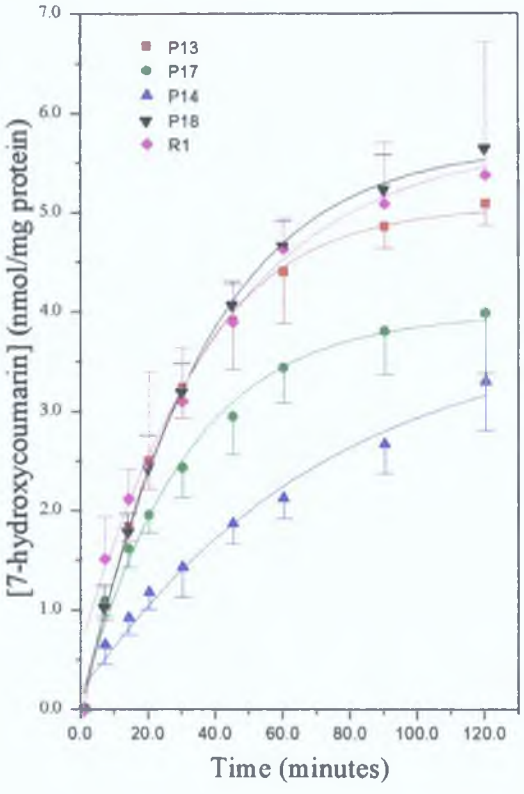


Figure 3.2.1.5. ***Plot of concentration of 7-hydroxycoumarin produced (nmol/mg protein) ± Standard Deviation (n=3) versus time (min) for the five microsome preparations. The profiles are as follows: P14-blue, P17-green, P13-red, R1-purple, P18-cyan. The samples were analysed by CE (see section 2.3.2.3. and 2.3.3.3.) and the concentrations determined from a plot of 7-hydroxycoumarin, prepared in the incubation solution, versus peak absorbance.***

Figure 3.2.1.6. is a comparison of HPLC and CE for the determination of 7-hydroxycoumarin formation. It can be seen that there is no significant difference between the results obtained by either method for the quantification of 7-hydroxycoumarin. The HPLC methods involve stopping the enzyme reactions by precipitating out the protein to minimise degradation of the HPLC system.

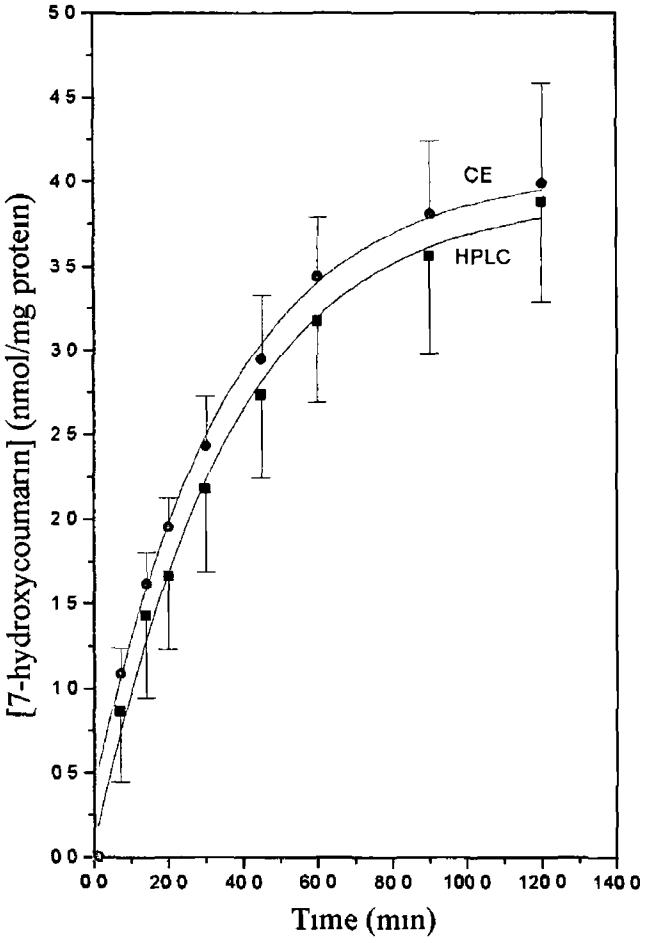


Figure 3 2 1 6 ***A comparison of HPLC and CE for the determination of 7-hydroxycoumarin after the in vitro metabolism of coumarin in the presence of human liver microsomal preparation Plot of 7-hydroxycoumarin (nmol/mg protein) ± Standard deviation (n=3) versus time for microsome preparation P17 metabolism study by HPLC (black) and CE (red)***

However, it was not necessary to precipitate out the protein in the CE method used as it involved injecting the incubation mixture directly onto the capillary The reaction is stopped by the separation of the individual components in the capillary After instantaneous separation they are thus, no longer able to interact further 7-hydroxycoumarin is separated after 0 9 min (Figure 3 2 1 3) using the CE method but it takes up to 12 min using the HPLC method of Egan and O'Kennedy (1992) to analyse

for 7-hydroxycoumarin Up to 500 injections were made onto the CE capillary during these studies without any visible degradation of capillary performance

3 2 2 1 3 NADP⁺ / NADPH Ratio Study

As part of the study we discovered that it was possible to monitor an integral part of the monooxygenase reaction i e the NADPH-dependent coumarin metabolism reaction To slow down the production of the NADPH from $NADP^+$ (structures of both NADPH and $NADP^+$ is given in Figure 3 2 1 4, and the migration times after CE separation is shown in Figure 3 2 1 2) the concentration of the glucose-6-phosphate-dehydrogenase was reduced to 0 1 units/ml as compared to 4 units/ml for the study of coumarin metabolism Figure 3 2 1 7 shows a plot of $NADP^+$ / NADPH absorbance ratio versus time for the incubation mixture and also for incubations in the absence of coumarin, or microsomes The $NADP^+$ / NADPH ratio was calculated from their respective absorbances at 210 nm Comparing the controls to the incubation complete incubation mixture, the $NADP^+$ / NADPH absorbance ratio changes as the cytochrome P450 utilises the NADPH for the metabolism of the coumarin However, other biological processes in the microsomes also utilise the NADPH in the controls Therefore, it was possible to analyse coumarin metabolism and the cofactor reaction which was shown to be necessary for the metabolism of coumarin Thus, CE can be used to monitor if drugs are metabolised using an NADPH system or not

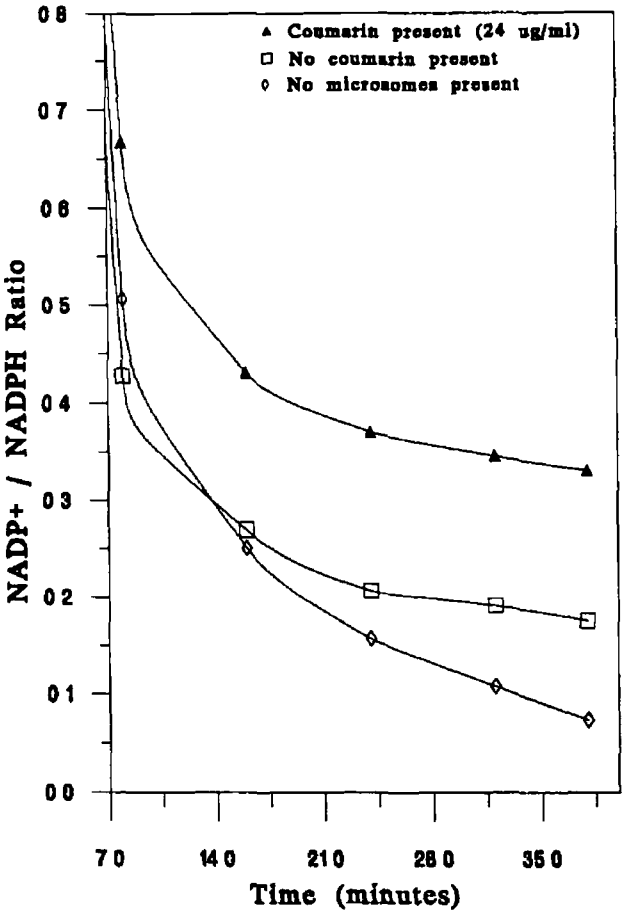


Figure 3 2.1 7 Plot of NADP⁺ / NADPH absorbance ratio versus time, for each of the controls and the incubation mixture The controls included the incubation mixture without coumarin or without microsomes The samples were analysed by capillary electrophoresis Separation was carried out on a 27 cm untreated silica capillary using 25 mM phosphate buffer (pH 7 0) and detection at 210 nm

3 2 2 2 Interspecies differences in coumarin metabolism in liver microsomes

3 2 2 2 1 Development of the CE separation

The choice of pH and electrolyte concentration was optimised to separate the components of the reaction mixture 50 mM phosphate buffer (pH 6 8) was found to be optimal, with a working current of 80 μamps The CE method used for the determination of coumarin 7-hydroxylase activity in the different species was only

slightly different from that used in the study using human microsomes The ionic strength and pH of the buffer as well as the applied voltage were altered

3 2 2 2 Coumarin Metabolism

To assess whether or not the microsomal preparation was still active it was necessary to carry out a series of controls Other side reactions occurring during the metabolism of coumarin to 7-hydroxycoumarin produced other species which migrated with coumarin and the peak at this time was seen to increase over time Coumarin acts as a neutral species at this pH and is seen to migrate with a neutral marker species, benzamide (see section 3 2 3 1) This was observed in the presence and absence of coumarin and was used as an indicator of metabolism It was not possible to initiate any coumarin metabolism without the presence of the NADPH regeneration system or the microsomes The series of controls prepared included the omission of the NADPH regeneration system, the absence of the liver microsomes, and the absence of coumarin It was not necessary to stop the reaction prior to the analysis as the application of the high voltage separates the individual components of the reaction mixture, thus, preventing further reaction

A 10 µl aliquot was removed at the specified time and added into a microvial, from which it was analysed by the capillary electrophoresis system The separation observed was similar to that shown in Figure 3 2 1 3 The analysis of such a sample by HPLC system would, in time, cause degradation of the injection system, column and fouling of the detector No deterioration in capillary performance was observed over the period of the study The regeneration of the surface between every run also aided in maintaining the reliability of the method There was some inter-day variability in the migration times for the different components This was however, attributed to the slight variations in preparing the electrolyte buffer but was always less than ±10%

Coumarin 7-hydroxylation activity disappears if the microsomes are not stored correctly or mishandled during the *in vitro* assay Table 3 2 1 3 shows the coumarin 7-hydroxylase activities (pmol / mg protein / min) of the different species ± standard

deviations (n=3) (This data is also represented graphically in Figure 3 2 1 8) The activity of the microsomes from the cynomolgus monkey are similar to the human microsomal preparations studied The other species, however, are substantially less active as compared with the human samples

Species	Coumarin 7-hydroxylase Activity[1] ± Standard Deviation (n=3) (pmol/mg protein/min)
Rabbit	180 0 ± 16
Monkey	616 0 ± 8
Bovine	110 ± 8
Porcine	nq[2]
Rat	nq
Mouse (CD1)	nq
Mouse (Sch)	nq
Gerbil	152 0 ± 29
Dog	nq
Human microsomal preparation	
P13	867 0 ± 107
P14	415 0 ± 45
P17	653 0 ± 85
P18	905 0 ± 54
R1	909 0 ± 84

1 The coumarin 7-hydroxylase activity was determined at 45 minutes

2 nq Not quantifiable It was not possible to determine the coumarin-7-hydroxylase activity in these liver samples as the limit of quantification of 7-hydoxycoumarin was 6 2 nmol/ml

Table 3 2 1 3 Coumarin 7-hydroxylase activity in microsomal preparations as determined by CE (see section 2 3 2 3 and 2 3 3 4 The data is given as the species with the respective coumarin 7-hydroxylase activity (pmol produced per mg of protein per min) ± the mean activity (n=3) The results were calculated from a plot of 7-hydroxycoumarin, prepared in the incubation mixture, versus peak absorbance

Steensma *et al* (1994) found that coumarin 7-hydroxylase activity in cynomolgus monkey liver was greater than that observed in their human liver However, they studied activity in precision-cut liver slices and not in microsomal preparations Pelkonen *et al* (1993) found coumarin 7-hydroxylase activity in human, and rabbit liver, and very low levels in rat liver However, their activity was based on crude homogenates It is not possible to compare the activity, determined by the method

above and to that determined by other methods, as liver preparations, substrate concentrations, incubation mixtures, etc vary from assay to assay in different laboratories However, some of the results for activities observed in other studies (Table 3 2 1 1) for microsomal metabolism studies were similar to the results determined by CE

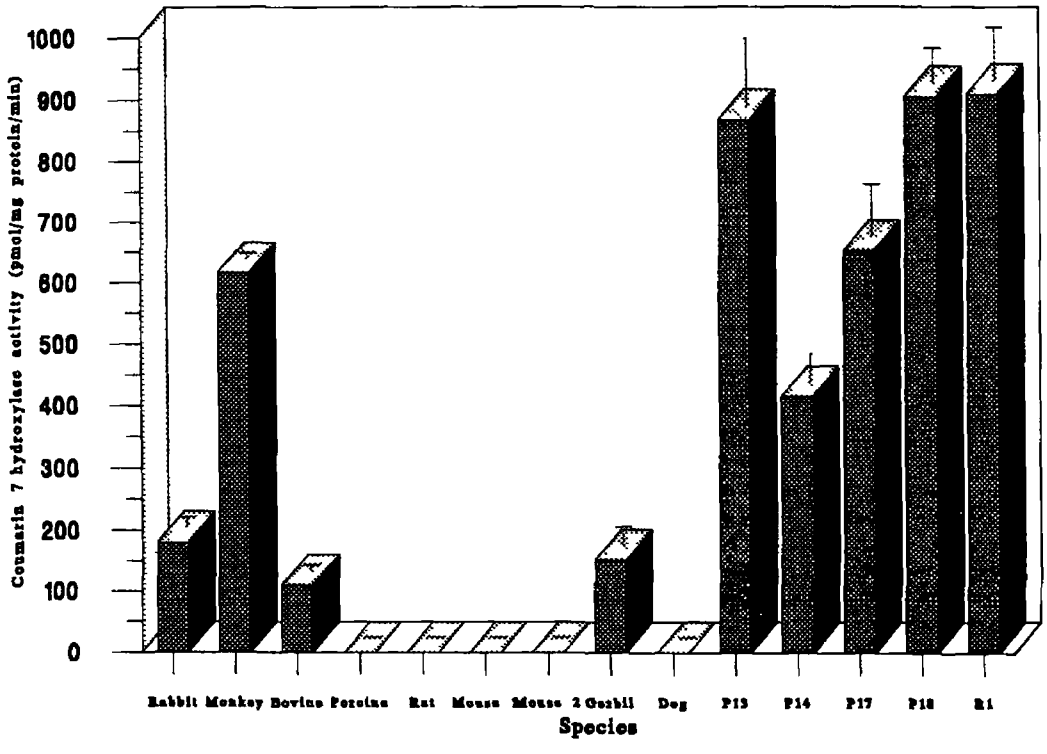


Figure 3 2 1 8 Graphical representation of coumarin-7-hydroxylase activity in microsomes from various species as determined by capillary electrophoresis The data is represented as the species with its mean respective activity (pmol/mg protein/min) ± standard deviation (error bars)

The human microsomal preparations had a variability between 415 0 and 909 0 pmol/ mg protein / min as compared to the range observed by Pelkonen *et al* (1996) of between 100 and 5000 pmol/mg protein/min The activity of the microsomes, determined by CE, were in this region Lake *et al 's* (1995) study yielded an activity of 999 ± 49 pmol / mg protein / min The results obtained by CE give a clear indication of

interindividual variability as do Pelkonen *et al 's* (1996) The other activities determined were generally lower than those observed by other groups However, comparison of the results from different groups in different laboratories indicated a large variability within species in coumarin 7-hydroxylation activities e g cynomolgus monkey microsomes had activities between 1780 and 3470 pmol/mg protein/min whereas the activity determined by our CE method was 616 0 pmol/mg protein/min Thus, it is not possible to determine any absolute value for coumarin 7-hydroxylation activity in any species but only to assess interindividual and interspecies mean variations

The observed differences in metabolic rates between species can be due to differences in the cytochrome P450 substrate affinity (K_m) or its maximal rate of product formation (V_{max}) These both depend on the evolution of the particular species The CE method developed will allow kinetic analysis of the P450 enzyme activity involved in the hydroxylation of coumarin, i e to ascertain K_m and V_{max} values It was not determined if the metabolic reaction monitored followed Michaelis-Menten kinetics

It has clearly been shown that different species do not predominantly metabolise coumarin to 7-hydroxycoumarin Therefore, to assess the effect on humans an appropriate model for coumarin metabolic studies must be chosen and from our study, cynomolgus monkey would be an appropriate and relevant model species for studying coumarin metabolism and toxicity

3 2 3. Glucuronidation (a short review)

The uridine diphosphate (UDP) glucuronyl transferases (also called glucuronosyltransferases) (UDPGT's) are a collection of enzymes that are membrane-bound and responsible for the 'glucuronidation' of millions of biological compounds and drugs The UDPGT's act by transferring the glucuronic acid moiety of UDP-glucuronic acid to compounds that have functional groups containing oxygen, nitrogen, sulphur and carbon (Figure 3 2 3 1) Figure 3 2 3 1 shows a schematic representation of the biological reaction to form a glucuronide conjugate of many compounds, endogenous and exogenous By the action of the glucuronyl transferase a hydrogen ion is displaced, from the substrate, and replaced with the glucuronic acid moiety forming the β-D-glucuronide conjugate A molecule of UDP is also formed Kaspersen and van Boeckel (1987) give a review on the chemical synthesis of glucuronide conjugates

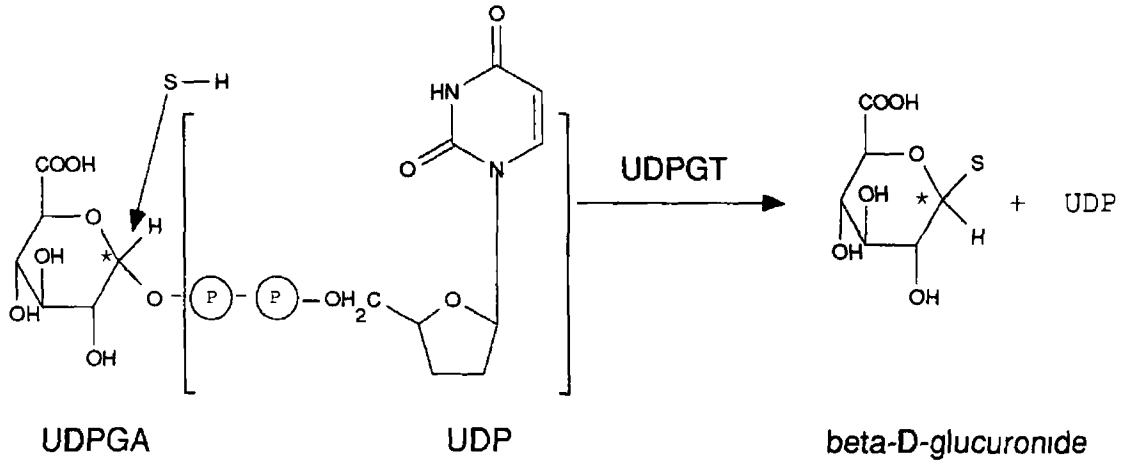


Figure 3 2 3 1 Glucuronidation of a substrate (H-S) by uridine diphosphate (UDP) glucuronyl transferases (* = the anomeric carbon atom, UDPGA = UDP-glucuronic acid) The substrate adds in to the glucuronic acid at the '*', displacing a 'H' which reacts forming UDP and the glucuronide conjugate (Reaction scheme taken from Kroemer and Klotz, 1992)

Elimination is facilitated by increasing the polarity of molecules, in essence making them less active and more readily soluble in bile and urine 'Detoxification' is generally accepted as occurring in two steps - step 1 is reacting the molecule to give it a

functional side group i e an -OH, -NH_2, or -COOH, that can react further - step 2 is conjugation to a highly polar molecule such as glucuronide, sulphate, or acetate Detoxification is the general acceptance of the function of glucuronidation and as a product of this it facilitates elimination In general, glucuronides have high water solubility, low protein binding and small volumes of distribution, and, thus, are rapidly eliminated from the body in the urine They are thus, disregarded by many in pharmacokinetic and pharmacodynamic studies and are assumed to be inactive pharmacologically However, this may be applicable to a large array of drug-glucuronides, but there are some notable exceptions There is now more interest in the glucuronide conjugates of many xenobiotics (Dutton, 1980, Kroemer and Klotz, 1992, Olson *et al* , 1992, Kauffman, 1994) in clinical observed effects and in therapy (see below)

The effect of some drugs is determined directly by its glucuronide form Indirect effects may also be due to glucuronides (e g at a particular site the parent drug may be released from its glucuronide form and allowed to have some effect - this is usually assisted by enzymatic cleavage by β-glucuronidase or covalent protein binding)

The best documented glucuronide with direct clinical effects are those of morphine (Kroemer and Klotz, 1992) *N-O*-glucuronides of *N*-hydroxypentacetin, estriol-17-β-(β-D-glucuronide), estriol-16-α-(β-D-glucuronide) and testosterone-17-β-(β-D-glucuronide) have all been shown to have a direct clinical effect (Kroemer and Klotz, 1992) Indirect effects may also be attributed to glucuronides One way that the glucuronide may play a clinical role is in a systemic cycle where the parent drug and glucuronide conjugate may continuously conjugate and deconjugate This may occur at various locations in a particular environment (Figure 3 2 3 2)

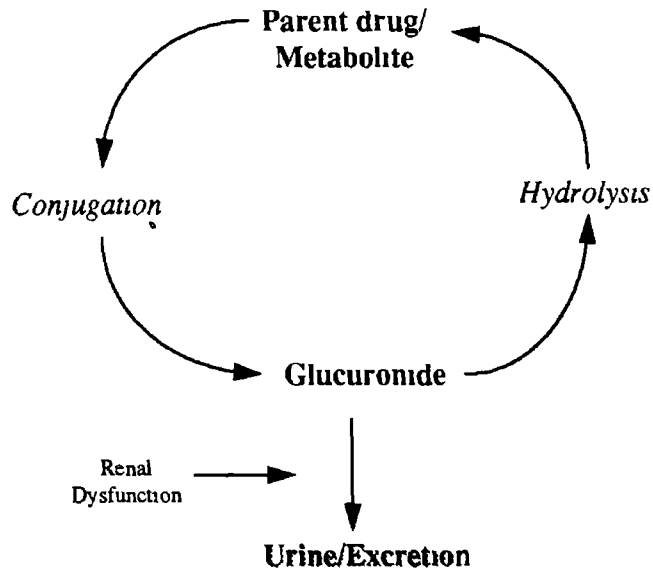


Figure 3 2 3 2 The proposed systemic cycling showing the reactivity of glucuronides The parent compound is recruited from the glucuronide by enzymatic cleavage Excretion of the glucuronide may be hampered by kidney dysfunction thus allowing it to remain in circulation Casley-Smith and Casley-Smith (1986), suggested that due to the active transport shown to exist for glucuronides, that it is possible that 7-hydroxycoumarin-glucuronide is transported into the cells Glucuronidases present in the cell reconvert the glucuronide to the 7-hydroxycoumarin After it has exerted its pharmacological action, it might then be reglucuronidated before excretion (The figure is taken from Kroemer and Klotz, 1992)

Intramolecular rearrangement of glucuronides may lead to glucuronide resistance It is known that ether glucuronides are very stable, however, ester glucuronides undergo structural rearrangement to form β-glucuronidase resistant forms Another indirect effect may involve the covalent binding of the glucuronides to proteins or the removal of the glucuronic acid moiety at a site on a protein This effect was observed for zomepirac and oxaprozin Zomepirac is an anti-inflammatory drug with analgesic effects (Langendijk *et al* , 1984) Zomepirac-glucuronide is its major metabolite in man In the case of zomepirac it was believed that the zomepirac-glucuronide after undergoing isomerisation allows ring-opening The aldehyde moiety formed, forms an imine linkage with a lysine or terminal amino group of a protein which can undergo further rearrangement After the death of several people, who were being treated with

zomepirac, it was withdrawn The effect was attributed to this protein binding Langendijk *et al* (1984) reported that they had observed an isomeric conjugate of zomepirac-glucuronide in the urine of patients and attributed it to acyl migration These direct and indirect effects observed may have an important role with respect to drug toxicity and immune response and also to the inaccuracies in glucuronide concentration determinations if β-glucuronidase hydrolysis is used in the analysis of the compound Several other examples are presented by Dutton (1980) and Kroemer and Klotz (1992)

The principle site of glucuronidation in vertebrates is the liver In liver cells it occurs in the endoplasmic reticulum and to a lesser extent in the cytoplasm, the mitochondria and the nuclear envelope (Dutton, 1980) The plasma membrane showed negligible UDPGT activity Mechanical disruption, the addition of surfactants, temperature effects, storage, solvents, chelating agents, trypsin, phospholipids, metal ions, and ATP/AMP are some of the different species investigated for studying the activation and inhibition of UDPGT (Dutton, 1980) The liver is not the only site of glucuronidation Table 3 2 3 1 show the activity of UDPGT's in various extrahepatic tissue for the substrate 4-methylumbelliferone

Tissue	UDPGT Activity (nmol substrate glucuronidated / gram wet weight)	UDPGT Activity (nmol substrate glucuronidated / gram whole organ)
Liver	460 0	3200 0
Duodenal mucosa	260 0	96 0
Kidneys	150 0	120 0
Adrenal glands	170 0	63 0
Spleen	30 0	35 0
Lungs	28 0	34 0
Thymus	19 0	8 8
Heart	1 4	1 1
Brain	0 8	1 3

Table 3 2 3 1 Uridine diphosphate glucuronyl transferase (UDPGT) activities in rat tissue for 4-methylumbelliferone The data is presented as the UDPGT activity in nmol of 4-methylumbelliferone glucuronidated per gram wet weight and per gram of the whole organ (Table taken from Dutton, 1980)

The liver was the principle site for glucuronidation for 4-methylumbelliferone However, activity was UDPGT activity for the glucuronidation of 4-methylumbelliferone was observed in all other tissue types Activity was lowest in the heart and in the brain Duffy *et al* , (1996) reported on the *in vitro* glucuronidation of 7-hydroxycoumarin in rabbit tissue sample homogenates Table 3 2 3 2 are the concentrations (pmol) of 7-hydroxycoumarin-glucuronide produced per minute per milligram of protein in the homogenates

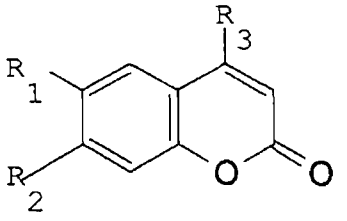
Tissue	UDPGT activity
	(pmol 7-hydroxycoumarin-glucuromde produced per min per milligram of protein)
Liver	2300
Kidney	220
Bladder	140
Large intestine	7 8
Lung	0
Spleen	0
Fat	0
Heart	0

Table 3 2 3 2 Uridine diphosphate glucuronyl transferase (UDPGT) activity in rabbit tissue homogenates as determined by HPLC The data is presented as the tissue source and the activity in pmol of 7-hydroxycoumarin-glucuronide produced per minute per milligram of protein (Table taken from Duffy et al , 1996)

A full length cDNA clone for a human hepatic UDPGT was isolated from a human liver cDNA library and stably expressed in human embryonic kidney 293 [HK293] cells (Green *et al* , 1994) The UDPGT activity for over 100 compounds was tested in the presence of the cells with the UDPGT expressing protein Glucuronyl transferase activity was observed toward several classes of xenobiotics, including simple phenolic compounds, 7-hydroxylated coumarins, flavanoids, anthraquinones and for some estrogens and androgens

Table 3 2 3 3 is a list of the coumarins with the respective enzyme activities of the transfected HK293 cells The structures of each of the derivatives are also given

Activities were determined by assaying for uridine diphospho-[U-^{14}C]-glucuronic acid The reaction solution was treated with ethanol to cause protein precipitation After centrifugation the residue was dissolved into water and analysed by thin layer chromatography on 0 5 mm silica gel 60 T_{254} plates



Coumarin derivative	R_1	R_2	R_3	Enzyme activity pmol/min/mg protein
4-hydroxycoumarin	H	H	OH	nd
Umbelliferone	H	OH	H	72
4-methylumbelliferone	H	OH	CH_3	170
Esculetin	OH	OH	H	110
Scopoletin	OCH_3	OH	H	170

Table 3 2 3 3 Glucuronidation of coumarin derivatives by transfected HK293 cells stably expressing UGT2B15 The substrate concentrations were UDP-glucuronic acid 0 5 mM, and coumarin derivative 0 1 mM nd - indicates that no glucuronidation was detected (Table taken from Green et al , 1994)

Conway *et al* , 1984, investigated the rates of glucuronidation in periportal and pericentral regions of the liver lobule using micro-light guides placed on the regions of the liver surface The fluorescence of free 7-hydroxycoumarin was determined and related to the glucuronidation rate by determining the decrease in fluorescence over time under various conditions The maximal rates of glucuronidation in periportal and pericentral regions of the liver lobule were 9 6 and 3 5 µmol/g/hr, respectively The details of the analysis is covered in section 1 3 1 2

High performance liquid chromatography (Langendijk *et al* , 1984, Musson *et al* , 1985, Lehr *et al* , 1987, Goresky and Gordon, 1990, and Vree and Kolmer, 1992), and enzyme-linked immunosorbent assay (Mitsuma *et al* , 1989, Lewis *et al* , 1990, Sailstad and Findlay, 1991, and Stabenfeldt *et al* , 1991) are the general methods used for the determination of glucuronides in urine and plasma samples The methods referenced above are for a range of different drug-glucuronides including - penbutolol, oestrogen, bilirubin, zomepirac, diflunisal, probenecid, lamotrigine, and pregnanediol Knight and Skellern, (1980), and To and Wells, (1984), both used HPLC for the determination of UDPGT activities Capillary electrophoresis has also been used for the determination of glucuronides in urine (Wernly *et al* , 1993)

Section 3 2 3 1 following describes the use of capillary electrophoresis for determining the glucuronidation of 7-hydroxycoumarin A crude liver homogenate was prepared and the glucuronyl transferase activity was determined by quantitating the production of 7-hydroxycoumarin-glucuronide in the reaction solution

3 2 3 1 Glucuronidation of 7-hydroxycoumarin in vitro

In man the majority of coumarin administered is excreted as 7-hydroxycoumarin-glucuronide (Rautio *et al* , 1992, Egan and O'Kennedy, 1993) The CE method utilised (see section 2 3 1 1) for the determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine was applied to the determination of 7-hydroxycoumarin-glucuronide in an *in vitro* metabolic assay

Separation of 7-hydroxycoumarin-glucuronide from endogenous species present in the metabolic matrix and from 7-hydroxycoumarin, is achieved in less than 7 minutes (Figure 3 2 3 1 1) At 1 min no appreciable 7-hydroxycoumarin-glucuronide was observed, but after 12 min the UDPGT had already started to produce 7-hydroxycoumarin-glucuronide At 47 and 82 min there was a visible increase in the 7-hydroxycoumarin-glucuronide (peak C) content in the matrix The reaction proceeded in a linear manner for the first 70 min The mean rate of reaction (Figure 3 2 3 1 2 and Table 3 2 3 1 1) for the first 70 min was found to be 3 09 ± 0 13 nmol of glucuronide

produced per minute per mg of protein (n=4) After 70 min the enzyme efficiency slows down and after 180 min (results not shown) no more 7-hydroxycoumarin-glucuronide is formed The reaction rate was slowed due to substrate limitation, enzymatic deactivation, or β-glucuronidase deconjugation in the reaction solution or a combination of these factors

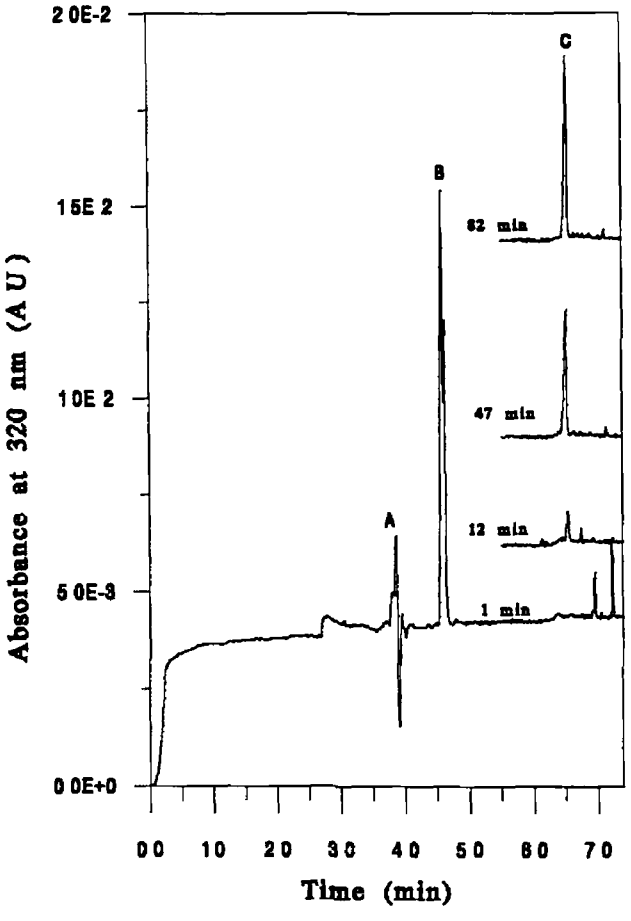


Figure 3 2 3 1 1 CE electropherogram of metabolic solution as outlined in section 2 3 2 4 and 2 3 3 5 Separation was carried out in 90 % 100 mM phosphate buffer, 11 mM deoxycholic acid (sodium salt) 10 % acetonitrile, on a 47 cm uncoated silica capillary, at 20 kV, with detection of the analytes at 320 nm It shows an overlay of electropherograms of samples taken at 1 min, 12 min, 47 min and 82 min, respectively The peaks are (A) solvent front containing all neutral components, (B) Magnesium chloride and 7-hydroxycoumarin (shoulder) and (C) 7-hydroxycoumarin-glucuronide Electropherograms for 12 min, 47 min and 82 min are shown in offset to illustrate the increase in peak area and are displayed between 5 5 min and 7 5 min only The remainder of the electropherogram was typical of the 1 min profile

In the absence of enzyme, 7-hydroxycoumarin or UDPGA no 7-hydroxycoumarin-glucuronide was produced It was possible to monitor the breakdown of the glucuronide conjugate over time to 7-hydroxycoumarin, when the undenatured protein solution was used as the matrix for the standards The limit of detection for 7-hydroxycoumarin-glucuronide was 2 µg/ml with a linear range of 0 - 100 µg/ml Percentage relative standard deviations for each standard peak area were all below 10%

Time	7-hydroxycoumarin-glucuronide Concentration ± Standard Deviation (n=4)	% Relative Standard Deviation
(min)	(µM)	
1	0	0
12	35 1 ± 4 3	12 3
23	72 8 ± 4 6	6 3
35	112 3 ± 6 0	5 3
47	140 9 ± 11 5	8 2
58	189 4 ± 4 2	2 2
70	207 6 ± 2 0	1 0
82	222 1 ± 7 9	3 6

Table 3 2 3 1 1 Results for the 7-hydroxycoumarin-glucuronide concentration from the study into the in vitro production of 7-hydroxycoumarin-glucuronide from 7-hydroxycoumarin The results are given as the time at sampling and the respective 7-hydroxycoumarin-glucuronide concentration ± standard deviation (n=4) determined by CE, and the % relative standard deviation

The CE method developed was found to be very fast and reliable for the direct determination of 7-hydroxycoumarin-glucuronide as the *in vitro* metabolite of 7-hydroxycoumarin and UDPGA by the enzymatic action of UDPGT The method was reproducible with excellent precision and accuracy No sample-clean up was necessary and injection of the neat mixture onto the capillary allows results to be obtained virtually in 'real-time' without any interference from errors due to sample preparation (extraction steps, precipitation steps etc) The method would be ideal for the investigation of UDPGT inhibitors or activators and for the assessment of glucuronidation in other tissue

types besides the liver Thus, CE has been demonstrated to be applicable to the *in vitro* and *in vivo* determination of both Phase I and Phase II metabolic studies of coumarin

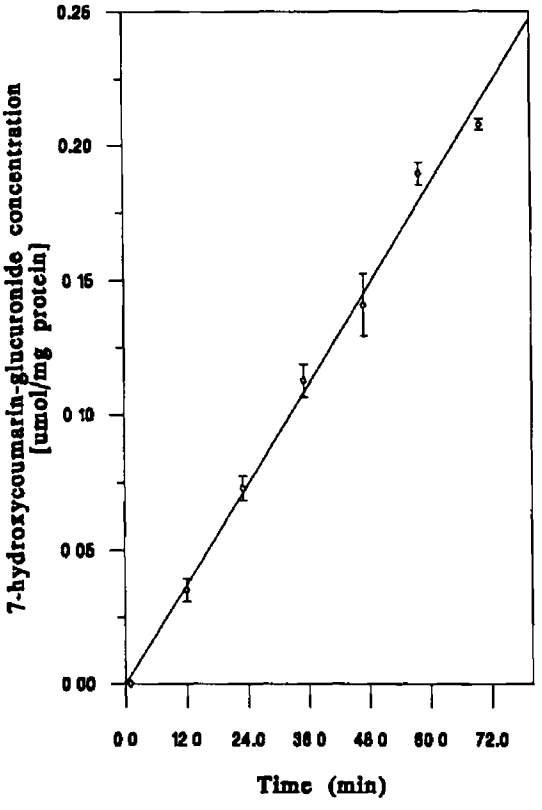


Figure 3 2 3 1 2 ***A plot of Time (min) versus mean 7-hydroxycoumarin-glucuronide concentration (n=4) (μmol/mg protein) from incubation assay study ± standard deviation (error bars) The equation of the line is y = -3 2E-4 + 3 1E-3 x with an r^2 = 0 99 The samples were analysed by CE as described in section 2 3 2 4 and 2 3 3 5 The concentrations were calculated from a plot of 7-hydroxycoumarin-glucuronide concentration, prepared in denatured enzyme solution, versus peak absorbance***

Summary

Capillary Electrophoresis was shown to be an excellent tool for the determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine and serum, either by direct injection or after extraction, evaporation and reconstitution CE was also shown to be very applicable for studying the *in vitro* metabolism of coumarin and 7-hydroxycoumarin Each of the methods developed were compared to HPLC and some urine samples were also analysed by ELISA (see section 5 4 1) There was no statistical difference between the results as determined by CE and any of the other methods to which it was compared

CHAPTER 4

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THE ANALYSIS OF COUMARINS

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4 0 AN INTRODUCTION TO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

4 0.1. Normal-phase HPLC

4 0 2 Partition-chromatography

4.0 3. Gel-permeation chromatography

4 0.4 Reverse-phase HPLC

4 0.5 Instrumentation

4.1 THE SIMULTANEOUS DETERMINATION OF COUMARIN, 7-HYDROXYCOUMARIN, AND 7-HYDROXYCOUMARIN-GLUCURONIDE IN HUMAN PLASMA, SERUM AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

4 1.1 Development of the HPLC separation

4 1.2 Limit of detection, linearity, precision and accuracy

4 1 3 Clinical and pharmacokinetic studies

4 1 3 1 Study 1 Analysis of plasma samples from volunteers who had been treated with 250 mg of coumarin

4.0 AN INTRODUCTION TO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

4 0 1 Normal-phase HPLC

Normal-phase chromatography, also known as adsorption chromatography, allows the separation of components based on differences both in their degree of adsorption by the adsorbent and solubility in the solvent used for separation The molecular structure of the compound to be separated governs these features The eluents in normal-phase chromatography are usually relatively non-polar organic solvents e g hexane There is usually a polar stationary phase Thus, the analytes are eluted off the HPLC system in order of increasing polarity, while retention decreases with increasing solvent polarity Compounds that are highly soluble in organic solvents, such as lipids and fat-soluble vitamins, are usually separated by normal-phase HPLC

4 0.2 Partition-chromatography

Partition-chromatography is used for the separation of a very wide range of biological compounds It is based on the partition of the solute between the liquid stationary phase and the mobile phase In normal phase partition-chromatography, the stationary phase is water supported by a matrix In reverse-phase partition chromatography, the stationary phase is a non-polar compound such as liquid paraffin supported by a matrix similar to those employed in normal phase systems (i e cellulose, starch or silicic acid)

4 0 3. Gel-permeation chromatography

Gel-permeation chromatography or size-exclusion chromatography, separates compounds on the basis of their molecular size Large molecules, outside the molecular weight 'cut-off' range of the gel, are excluded from the gel, as they cannot enter the pores of the stationary phase Elution occurs in order of decreasing size, the smallest molecules being eluted last

4 0 4 Reverse-phase HPLC

Reverse-phase chromatography is the opposite to normal-phase chromatography Thus, the mobile phase is more polar than the stationary phase The solute is retained mainly as a result of hydrophobic interactions between the solutes and the hydrophobic stationary phase surface Solutes are normally eluted in order of decreasing polarity (increasing hydrophobicity) and increasing the content of the non-polar component in the mobile phase, results in the decrease of the retention of the solutes Various additives can be incorporated into the mobile phase to give special selectivities to the separation Hence, reverse-phase chromatography may be extended to include ion-pairing, ionisation and ion-suppression forms of HPLC The typical stationary phase in reverse-phase HPLC is a silica surface with 18 carbon hydrophobic chains chemical coupled to it and mobile phases are typically based on methanol, water, acetonitrile, or tetrahydrofuran

4 0.5. Instrumentation

The basic equipment required for HPLC separation is a pump, sample introduction system, column, detector, waste collector and data analyst (computer or integrator) Figure 4 0 1 is a schematic representation of the system used for the development of the methods described in section 4 1 and 4 2 The separation and determination of coumarin and some of its derivatives by HPLC was described in section 1 2 2

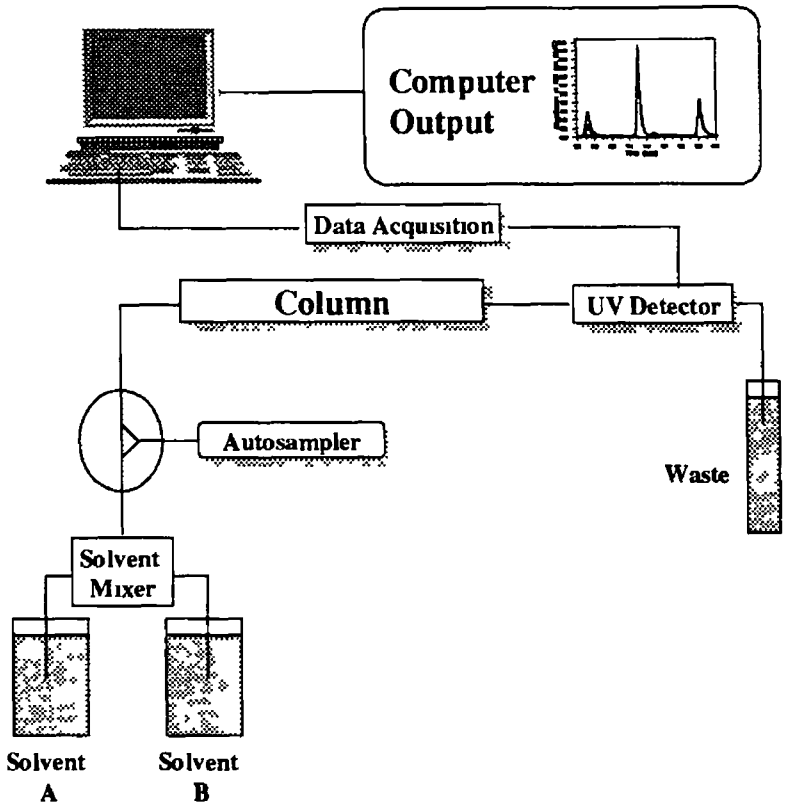

Figure 4 0 1 Schematic representation of HPLC system The system consisted of a dual pump/solvent delivery system and solvent mixer [module 126], an autosampler injection system [module 507], a reverse-phase silica based C18 column, a UV detector [module 166], a solvent waste bottle and data processing unit (computer-Dell Dimension 486) The system was controlled by System Gold™ software (version 8 0)

The sections following are the applications of HPLC for the determination of coumarin and some of its metabolites in a variety of matrices A HPLC method was developed and utilised for the direct determination of 7-hydroxycoumarin, coumarin, and 7-hydroxycoumarin-glucuronide in human plasma, serum and urine The method was also used for the determination of the *in vitro* production of 7-hydroxycoumarin-glucuronide, by uridine diphosphate glucuronyl transferase (UDPGT) in liver homogenate The method of Egan and O'Kennedy (1992) was utilised in the study of the metabolism of 7-hydroxycoumarin in patients with advanced malignancies

4.1. THE SIMULTANEOUS DETERMINATION OF COUMARIN, 7-HYDROXYCOUMARIN, AND 7-HYDROXYCOUMARIN-GLUCURONIDE IN HUMAN PLASMA, SERUM AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

A HPLC method was developed for the determination of the *in vivo* metabolites of coumarin and 7-hydroxycoumarin, i.e. coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide, in urine, plasma and serum (see section 2.2.3.2.). The HPLC method was applied to the determination of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in volunteers who had been treated orally with coumarin (urine and plasma samples) or 7-hydroxycoumarin (serum samples).

4.1.1. Development of the HPLC separation

The separation of coumarin, 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and the internal standard, 4-hydroxycoumarin (see Choice of internal standard in this section below) was optimised to give baseline resolution (Figure 4.1.1.) of the four compounds. This HPLC separation method (see section 2.2.3.2.) was found to be applicable to the analysis of each of the compounds in urine, plasma and serum. The method was initially developed with the standards spiked into water. Standards of coumarin, 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and 4-hydroxycoumarin were then spiked into each of the relevant biological fluids and the method was adjusted until the analytes were successfully resolved from any endogenous species present in the matrix of interest. However, interference from endogenous species present in urine, did not allow complete baseline separation of the analytes of interest from the interfering species.

The solvents used for the separation were selected because they had been successfully used previously by Egan and O'Kennedy (1992). However, when 7-hydroxycoumarin-glucuronide was analysed by the method of Egan and O'Kennedy (1992) it eluted in the void volume and thus, a method that would retard it on the column was sought. The

method of Egan and O'Kennedy (1992) was based on separation under isocratic conditions (see section 2 2 3 1) A separation based on gradient elution was developed and the method for separation is described in section 2 2 3 2 The gradient profile found to be most ideally suited for the separation of coumarin, 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and 4-hydroxycoumarin is shown in Figure 4 1 1

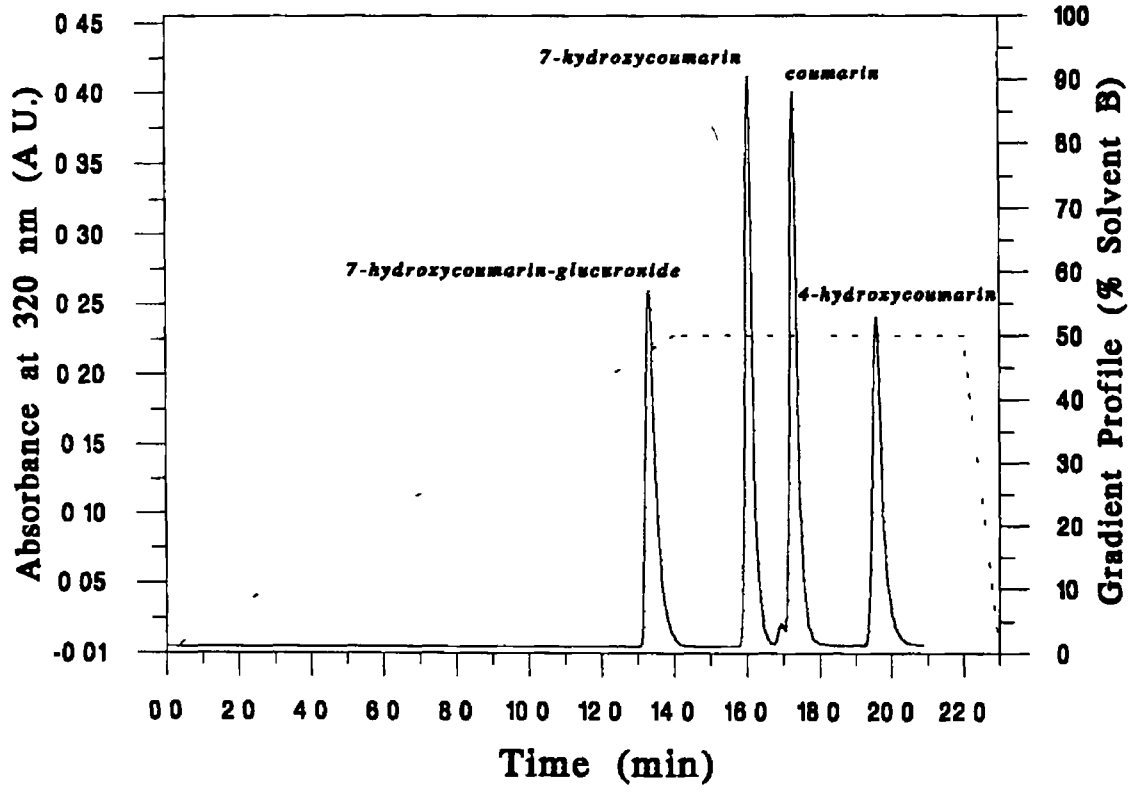

Figure 4 1 1 The chromatographic separation of 7-hydroxycoumarin-glucuronide (13 3 min), 7-hydroxycoumarin (16 0 min), coumarin (17 3 min), and the internal standard 4-hydroxycoumarin (19 6 min) prepared in water A chromatogram of time (min) versus absorbance at 320 nm (absorbance units) The gradient profile, represented as a percentage of solvent B (100 % methanol) versus time (min) is shown as a dashed line (- - - -) The sample was separated by reverse-phase HPLC on a C18 column with gradient elution (see section 2 2 3 2) and detection at 320 nm

Starting with 100 % solvent A (95 5 0 2 water methanol acetic acid, (v/v/v) and ramping up to 50 50 solvent A solvent B (100 % methanol) over 14 minutes gives excellent separation of the analytes and the internal standard However, if the percentage solvent of each changed more rapidly to a 50 50 solvent A solvent B, the samples were not fully resolved and if the rate was slowed the time factor for separation became too long After the gradient profile returned to 100 % solvent A (i e at 23 min) the absorbance was monitored until it returned to baseline zero At this point (about 9 min later) the system was then set to inject the next sample (this was assessed initially when the samples were prepared in water and subsequently in plasma, serum and urine)

Choice of internal standard

A range of compounds, mainly coumarins (o-coumaric acid, 7-amino-4-methyl coumarin, warfarin, coumarin-3-carboxylic acid and o-hydroxyphenylacetic acid), were investigated for their applicability as internal standards An internal standard is used to allow for differences in the physical properties of a series of sample solutions that contain the same analyte A fixed quantity of a pure substance is added to samples and standards alike The responses (e g absorbance) of the analyte and internal standard are determined, and the ratio of the two responses is calculated (i e absorbance analyte / absorbance internal standard) The response for the internal standard should be constant since the concentration of the internal standard is fixed If, however, one or more of the parameters that effect the measured responses vary, the responses of the analyte and the internal standard should be equally affected Thus, the response ratio should only be dependent on the analyte concentration A plot of the response ratio as a function of the analyte concentration yields a calibration curve from which unknown analyte concentrations may be calculated from their respective response ratio The internal standard must be added before measurement is made to allow for dissolution, mixing and any other reactions to occur The internal standard should be a substance, similar to the analyte, with an equally measurable signal that does not interfere with the response of the analyte The retention time of the internal standard must be within a range that makes it practical for its use 4-hydroxycoumarin proved to be the best candidate for internal standard It does not coelute with any other species, there is no interference

from other endogenous species in urine, plasma or serum, and it has a similar absorbance at 320 nm to the other analytes.

Figure 4.1.2. shows an overlay of the separation of the three analytes and the internal standard in serum and in blank serum. It is clearly seen that there is no interference from endogenous species present in the serum. This is also seen for the analysis of blank plasma and standards in plasma (result not shown).

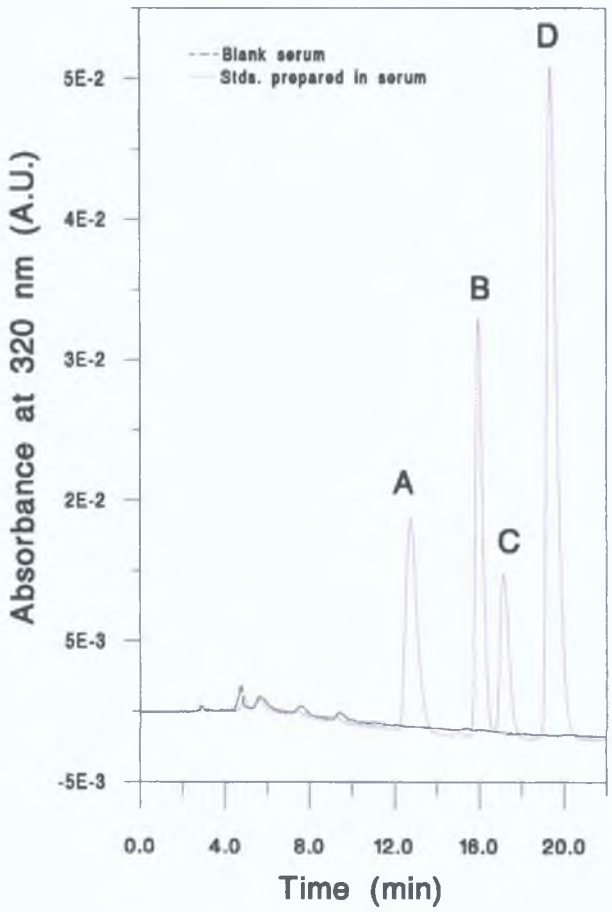

Figure 4.1.2. Chromatogram showing the overlay of the separation of (A) 7-hydroxycoumarin-glucuronide, (B) 7-hydroxycoumarin, (C) coumarin and (D) 4-hydroxycoumarin standards prepared in serum [purple], and blank serum [black]. The samples were separated by reverse-phase HPLC on a C18 column with gradient elution (see section 2.2.2.2.) and detection at 320 nm. Note: for all subsequent chromatograms each of the analytes and the internal standard is referred to as (A),(B),(C), and (D), respectively as in this Figure.

Figure 4.1.3. shows the separation of coumarin, 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and 4-hydroxycoumarin prepared in control urine and analysed by HPLC with gradient elution (see section 2.2.3.2.). The standards samples were prepared and analysed as outlined in section 2.2.2.2. and 2.2.3.2. The urine samples were analysed directly, except for the addition of the internal standard, or, if dilution in control urine was required to facilitate analyte concentration calculation. Protein precipitation was not utilised in sample preparation. The control urine was donated from a volunteer who had not been treated directly with coumarin or 7-hydroxycoumarin.

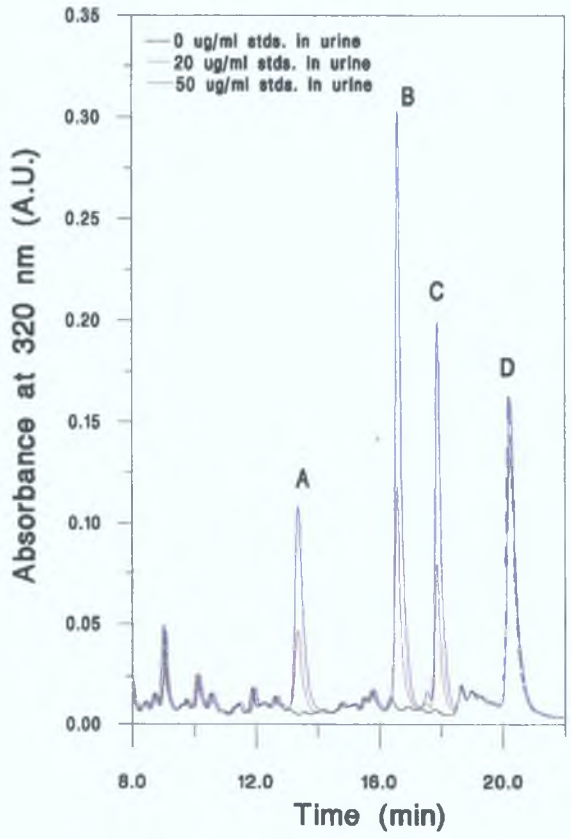

Figure 4.1.3. Overlay of the HPLC separation (8 min-22 min) of (A) 7-hydroxycoumarin-glucuronide, (B) 7-hydroxycoumarin, (C) coumarin and (D) 4-hydroxycoumarin standards (0 μg/ml [black], 20 μg/ml [red] and 50 μg/ml [blue]) prepared in urine and analysed as outlined in section 2.2.3.2. The samples were separated by reverse-phase HPLC on a C18 column with gradient elution and detection at 320 nm.

4 1 2 Limit of Detection, linearity, precision and accuracy

Plasma and serum standards

In both plasma and serum the limit of detection was 50 ng/ml for 7-hydroxycoumarin and 200 ng/ml for coumarin and 7-hydroxycoumarin-glucuronide The linear detection range was from 0 5 µg/ml to 100 µg/ml The accuracy and precision (Table 4 1 1) was determined for standards between 1 µg/ml and 100 µg/ml Correlation coefficients for any of the standard curves prepared were always better than 0 995 The absorbance ratio (absorbance of the analyte / absorbance of the internal standard) was plotted versus the concentration added to ascertain the precision of the method and the regression lines

The mean equation for absorbance ratio for the intra assay for each of the analytes were Coumarin - y = -0 01 + 0 01 X r^2 = 0 99, 7-hydroxycoumarin - y = -0 02 + 0 03 X r^2 = 0 99, 7-hydroxycoumarin-glucuronide - y = -0 01 + 0 02 X r^2 = 0 99
For the inter assay the mean standard curves were Coumarin- y = -2E-3 + 0 01 X, r^2 = 0 99, 7-hydroxycoumarin- y = 0 01 + 0 03 X, r^2 = 0 99, 7-hydroxycoumarin-glucuronide- y = 2E-4 + 0 02 X, r^2 = 0 99

The accuracy and precision and the results in Table 4 1 2 1 and Table 4 1 2 2 were calculated from these regression lines Table 4 1 2 1 and Table 4 1 2 2 give the mean concentration calculated ± standard deviation and the percentage relative standard deviations for both intra and inter assays The comparison of the calculated concentrations to the concentrations added and % relative standard deviations show that the method is both precise and accurate The percentage relative standard deviations in both the inter and intra assays are all below 10 % for each of the three analytes

Concentration Added	Mean calculated concentration *7-Hydroxycoumarin*	Mean calculated concentration *7-Hydroxycoumarin -glucuronide*	Mean calculated concentration *Coumarin*
	Conc ± St Dev (% RSD)	Conc ± St Dev (% RSD)	Conc ± St Dev (% RSD)
0	0	0	0
1 00	1 69 ± 0 01 (0 5 %)	1 39 ± 0 04 (2 6 %)	1 19 ± 0 03 (2 8 %)
5 00	5 38 ± 0 04 (0 7%)	4 97 ± 0 05 (0 9 %)	5 31 ± 0 24 (4 5 %)
10 00	9 60 ± 0 05 (0 5 %)	9 89 ± 0 12 (1 2 %)	9 70 ± 0 07 (0 7 %)
20 00	19 42 ± 0 05 (0 3 %)	19 82 ± 0 24 (1 2 %)	19 52 ± 0 11 (0 6 %)
50 00	49 30 ± 0 09 (0 2 %)	49 16 ± 0 11 (0 2 %)	49 43 ± 0 06 (0 2 %)
80 00	79 27 ± 0 23 (0 3 %)	82 20 ± 3 8 (4 6 %)	80 98 ± 0 16 (0 2 %)
100 00	102 5 ± 0 59 (0 6 %)	101 09 ± 0 9 (0 9)	102 4 ± 0 63 (0 6 %)

Table 4 1 2 1 Intra-assay precision and accuracy for the determination of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in plasma and serum (n=5) The samples were analysed by HPLC and the concentrations were calculated from the mean respective standard curves The data is presented as the mean calculated concentration ± standard deviation and the relative standard deviation about the mean

Concentration Added	Mean calculated concentration *7-Hydroxycoumarin*	Mean calculated concentration *7-Hydroxycoumarin -glucuronide*	Mean calculated concentration *Coumarin*
	Conc ± St Dev (% RSD)	Conc ± St Dev (% RSD)	Conc ± St Dev (% RSD)
0	0	0	0
1 00	0 89 ± 0 08 (8 8 %)	0 97 ± 0 04 (4 1 %)	1 29 ± 0 12 (9 6 %)
5 00	4 82 ± 0 27 (5 6 %)	4 82 ± 0 23 (4 7 %)	4 91 ± 0 37 (7 7 %)
10 00	10 16 ± 1 04 (10 0%)	9 92 ± 0 57 (5 8 %)	9 93 ± 0 67 (6 7 %)
20 00	20 31 ± 1 60 (7 9 %)	20 00 ± 0 79 (3 9 %)	20 22 ± 1 29 (6 4 %)
50 00	50 70 ± 2 05 (4 0 %)	51 13 ±1 86 (3 6 %)	49 10 ± 1 47 (3 0 %)
80 00	79 38 ± 3 62 (4 6 %)	78 70 ± 2 55 (3 2 %)	80 40 ± 2 78 (3 4 %)
100 00	100 10 ± 6 09 (6 1 %)	100 60 ± 3 82 (3 8 %)	100 08 ± 6 45 (6 4%)

Table 4 1 2 2 Inter-assay precision and accuracy for the determination of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in plasma and serum (n=5) The samples were analysed by HPLC and the concentrations were calculated from the mean respective standard curves The data is presented as the mean calculated concentration ± standard deviation and the percentage relative standard deviation about the mean is given in parenthesis

Urine standards

The limit of detection of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine was 500 ng/ml due to the high background signal as compared to plasma or serum The linear detection range was from 2 µg/ml - 100 µg/ml Tables 4 1 2 3 and 4 1 2 4 are the results for the intra- and inter- assay precision and accuracy for the determination of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine, by HPLC The comparison of the mean concentrations calculated to the concentrations added and % relative standard deviations show that the method is both precise and accurate

Concentration Added	**Mean calculated concentration** ***7-Hydroxycoumarin***	**Mean calculated concentration** ***7-Hydroxycoumarin -glucuronide***	**Mean calculated concentration** ***Coumarin***
	Conc ± St Dev (% RSD)	Conc ± St Dev (% RSD)	Conc ± St Dev (% RSD)
0	0	0	0
2 00	2 09 ± 0 04 (2 1 %)	2 01 ± 0 14 (7 0 %)	2 38 ± 0 08 (3 2 %)
5 00	5 05 ± 0 08 (1 6 %)	4 83 ± 0 31 (6 5 %)	5 10 ± 0 11 (2 1 %)
10 00	10 08 ± 0 26 (2 5 %)	10 25 ± 0 41 (3 9 %)	9 90 ± 0 17 (1 7 %)
20 00	19 70 ± 0 25 (0 5 %)	20 11 ± 0 26 (1 3 %)	19 60 ± 0 07 (0 3 %)
50 00	49 60 ± 0 17 (0 8 %)	50 80 ± 1 88 (3 7 %)	49 43 ± 0 30 (0 6 %)
80 00	81 32 ± 1 66 (2 0 %)	80 65 ± 0 96 (1 2 %)	80 27 ± 2 64 (3 3 %)
100 00	100 90 ± 3 91 (3 8 %)	99 00 ± 0 98 (1 0 %)	99 98 ± 3 61 (3 6 %)

Table 4 1 2 3 Intra-assay precision and accuracy for the determination of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine (n=5) The samples were analysed by HPLC and the concentrations were calculated from the mean respective standard curves The data is presented as the mean calculated concentration ± standard deviation and the relative standard deviation about the mean

Concentration Added	Mean calculated concentration *7-Hydroxycoumarin*	Mean calculated concentration *7-Hydroxycoumarin -glucuronide*	Mean calculated concentration *Coumarin*
	Conc. ± St. Dev. (% RSD)	Conc. ± St. Dev. (% RSD)	Conc. ± St. Dev. (% RSD)
0	0	0	0
2.00	1.6 ± 0.06 (4.1 %)	2.01 ± 0.06 (2.9 %)	1.97 ± 0.15 (7.7 %)
5.00	4.67 ± 0.12 (2.5 %)	4.86 ± 0.15 (3.1 %)	4.90 ± 0.24 (4.9 %)
10.00	9.70 ± 0.48 (5.0 %)	9.86 ± 0.23 (2.3 %)	9.56 ± 0.13 (1.4 %)
20.00	20.70 ± 0.37 (1.8 %)	20.82 ± 0.77 (3.7 %)	19.60 ± 0.94 (4.8 %)
50.00	49.96 ± 2.45 (4.9 %)	49.50 ± 0.89 (1.8 %)	51.30 ± 2.03 (3.9 %)
80.00	81.60 ± 3.29 (4.0 %)	79.75 ± 1.24 (1.6 %)	80.60 ± 6.65 (8.3 %)
100.00	98.80 ± 4.89 (4.9 %)	99.73 ± 3.73 (3.7 %)	99.72 ± 2.67 (2.7 %)

Table 4.1.2.4. Inter-assay precision and accuracy for the determination of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine (n=5). The samples were analysed by HPLC and the concentrations were calculated from the mean respective standard curves. The data is presented as the mean calculated concentration ± standard deviation and the relative standard deviation about the mean.

4.1.3. Clinical and pharmacokinetic studies

The method developed was applied to two separate studies:

Study 1: Plasma and urine samples were taken from each of three volunteers who had been treated with 250 mg of coumarin. The samples were analysed by HPLC.

Study 2: Serum samples were taken from patients with advanced malignancies (see section 4.3.) who had been treated with 7-hydroxycoumarin. The samples were analysed by HPLC.

4.1.3.1. Study 1: Analysis of plasma samples from volunteers who had been treated with 250 mg of coumarin

250 mg of coumarin was orally administered to each of three volunteers and plasma samples were taken at 0 h, 1 h and 4 h, respectively. The concentrations of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide, calculated from the standard

curve of mean absorbance ratio versus mean calculated concentration, is given in Table 4 1 3 1

Sample Time	Coumarin concentration	7-Hydroxycoumarin concentration	7-Hydroxycoumarin-glucuronide concentration
h	µg/ml	µg/ml	µg/ml
Volunteer 1			
0	NQ	0	0
1	NQ	ca 0 1	21 8
4	NQ	0	1 0
Volunteer 2			
0	NQ	0	0
1	NQ	ca. 0 2	12 7
4	NQ	0	0 8
Volunteer 3			
0	NQ	0	0
1	NQ	ca 0 1	9 6
4	NQ	0	0 5

Table 4 1 3 1 Table of HPLC results for the determination of 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and coumarin in plasma after the oral administration of 250 mg of coumarin The results were calculated from a standard curve of the mean absorbance ratio (Absorbance of analyte/absorbance of internal standard) versus mean concentration NQ - not quantifiable ca. - approximately- i e levels of drug were observed but their exact quantification was not possible as their absorbance values did not lie within the limits of the linear detection range

The samples were prepared as outlined under section 2 2 2 2 and analysed as outlined in section 2 2 3 2 Figure 4 1 3 1 shows the chromatograms from volunteer 2's plasma samples in overlay There is a majority of 7-hydroxycoumarin-glucuronide, as compared to 7-hydroxycoumarin, in the plasma sample after 1 h After 4 h the level of 7-hydroxycoumarin-glucuronide has decreased significantly After 1 h some free 7-hydroxycoumarin was observed in the plasma samples (ca 0 1 µg/ml) No coumarin was detected in the plasma after its oral administration From these results it was shown that coumarin enters the blood circulation, predominantly as (>98 % of the coumarin

observed) 7-hydroxycoumarin-glucuronide with some free unconjugated 7-hydroxycoumarin (ca 1 %)

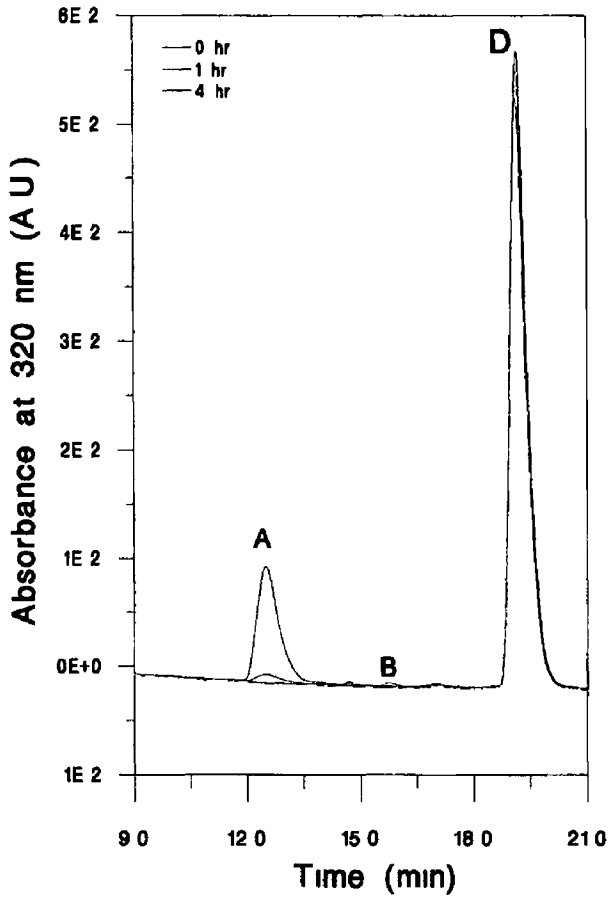

Figure 4 1 3 1 Chromatogram showing the overlay of volunteer 2's plasma samples after the administration of coumarin p o It shows the separation of (A) 7-hydroxycoumarin-glucuronide, (B) 7-hydroxycoumarin and (D) 4-hydroxycoumarin at 0 h [black], 1 h [red] and 4 h [blue], respectively The samples were analysed by reverse-phase HPLC on a C18 column with gradient elution (see section 2 2 3 2) and detection at 320 nm.

4 1 3 2 Study 1 Analysis of urine samples from volunteers who had been treated with 250 mg of coumarin

Each of three volunteers were given 250 mg of coumarin orally and urine samples were taken at 0 h, 2 h, 6 h, 10 h, 14 h, and 24 h, respectively The urine samples were diluted where necessary with control urine to ensure that the concentration of 7-hydroxycoumarin or 7-hydroxycoumarin-glucuronide was within the limits of the standard curve i e 2 µg/ml - 100 µg/ml Figure 4 1 3 2 shows the an overlay of two chromatograms for volunteer 2's urine samples taken at 0 h and 10 h, respectively

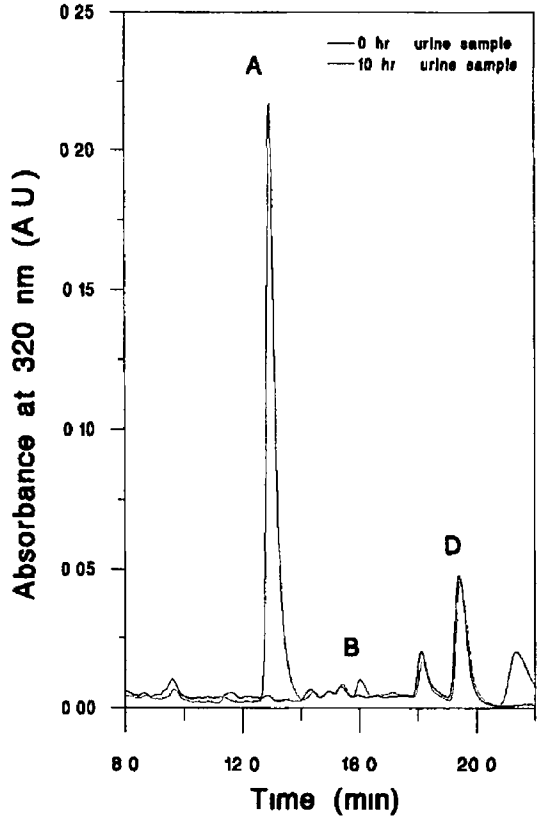

Figure 4 1 3 2 Overlay of volunteer 2's urine sample analysed by HPLC It shows the overlay of the urine sample at 0 h [black] and at 10 h [red] after the oral administration of 250 mg of coumarin The separation of (A) 7-hydroxycoumarin-glucuronide (B) 7-hydroxycoumarin and (D) 4-hydroxycoumarin The samples were analysed by reverse-phase HPLC on a C18 column with gradient elution (see section 2 2 3 2) and detection at 320 nm.

From the chromatogram, it is seen, that there is an excess of 7-hydroxycoumarin-glucuronide as compared to 7-hydroxycoumarin The concentrations of 7-hydroxycoumarin, coumarin and 7-hydroxycoumarin-glucuronide are given in Table 4 1 3 2 Up to 98 % of the 7-hydroxycoumarin excreted was excreted as the glucuronide conjugate The samples were also analysed by capillary electrophoresis (see sections 2 3 2 1 , 2 3 2 2, 2 3 3 1 , 2 3 3 2 , and 3 1 1) and the results determined by CE and HPLC were compared (Table 4 1 3 3) There was up to ± 11 % difference between the results from either method Up to 83 % of the coumarin administered was excreted as 7-hydroxycoumarin (free and conjugated)

Sample Time	Coumarin concentration	7-Hydroxycoumarin concentration		7-Hydroxycoumarin-glucuronide concentration	
h	µg/ml	µg/ml	µM	µg/ml	µM
Volunteer 1					
0	NQ	0	0	4 5	13 3
2	NQ	1 8	11 1	226 7	670 5
6	NQ	0	0	105 5	312 0
10	NQ	0	0	20 9	61 8
14	NQ	-	-	-	-
24	NQ	0	0	4 7	13 9
Volunteer 2					
0	NQ	0	0	0	0
2	NQ	60 3	372 2	2954 6	8738 8
6	NQ	16 6	102 5	880 5	2604 4
10	NQ	0	0	109 2	322 9
14	NQ	0	0	35 6	105 3
24	NQ	0	0	6 8	20 1
Volunteer 3					
0	NQ	0	0	0	0
2	NQ	39 3	242 6	3002 6	8880 8
6	NQ	5 3	32 7	680 8	2013 6
10	NQ	0	0	61 7	182 5
14	NQ	0	0	9 2	27 2
24	NQ	0	0	1 7	5 0

Table 4 1 3 2 HPLC results for the determination of 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and coumarin concentration (µg/ml and µM) in urine after the oral administration of 250 mg of coumarin. The analyte concentrations (µg/ml) were calculated from the standard curve of the mean absorbance ratio versus mean calculated concentration

There was a large variability between the three volunteers in their ability to excrete coumarin Only the excretion of coumarin as coumarin, 7-hydroxycoumarin or 7-hydroxycoumarin-glucuronide was assessed

Sample Time	7-OHC excreted (mg)			% Coumarin excreted as 7-hydroxycoumarin (free or conjugated) (% of dose administered)		
h	HPLC method	CE method 1	CE method 2	HPLC method	CE method 1	CE method 2
Volunteer 1						
0	1 9	0	ND	0 7	0	ND
2	40 2	52 9	ND	14 5	19 1	ND
6	20 2	25 6	ND	7 3	9 2	ND
10	2 9	2 8	ND	1 1	1 0	ND
14	NS	NS	ND	NS	NS	ND
24	0 9	0	ND	0 3	0	ND
Total	66 1	81 3	ND	23 8	29 3	ND
Volunteer 2						
0	0	0	0	0	0	0
2	88 5	107 4	110 6	31 9	38 7	39 9
6	30 7	36 4	40 9	11 1	13 1	14 7
10	15 6	13 3	17 7	5 6	4 8	6 4
14	1 7	1 6	2 6	0 6	0 6	0 9
24	1 7	0	1 7	0 6	0	0
Total	138 2	158 7	173 5	49 8	57 2	62 5
Volunteer 3						
0	0	0	2 2	0	0	0
2	133 2	153 4	149 3	48 0	55 3	53 8
6	65 7	67 7	69 8	23 7	24 4	8 8
10	8 9	6 5	8 7	3 2	2 3	0 8
14	1 8	2 2	1 4	0 6	0 8	0 5
24	0 3	0	0	0 1	0	0
Total	209 9	229 8	231 4	75 7	82 8	83 4

Table 4 1 3 3 7-hydroxycoumarin concentrations and % coumarin excreted as 7-hydroxycoumarin (free and conjugated) as determined by HPLC and CE 7-hydroxycoumarin (mg) excreted was calculated from total 7-hydroxycoumarin concentration [free and conjugated 7-OHC excreted (μg/ml)] X urinary volume collected (ml) 7-hydroxycoumarin excreted (mg) was related to the coumarin administered by multiplying it by their molar ratio (0 90) i e the % coumarin excreted was calculated from the total 7-OHC excreted (mg) X molar ratio (0 90) / coumarin administered (250 mg) X 100/1 The samples were prepared and analysed by HPLC method (see section 2 2 2 2 , and 2 2 3 2), by CE (Method 1, see section 2 3 2 1 and 2 3 3 1) and by CE (Method 2, see section 2.3 2.2. and 2 3 3 2) ND indicates that the sample concentrations were not determined and NS indicates that no sample was available for analysis

Volunteer 1 excreted ca 24 % of the coumarin administered as free or conjugated 7-hydroxycoumarin Volunteer 2 excreted ca 50 % and volunteer 3 excreted ca 76 % The presence of other coumarin metabolites in the urine samples was not determined Thus, the coumarin may have been excreted but in a different form, e g o-hydroxyphenylacetaldehyde or 3-hydroxycoumarin or 7-hydroxycoumarin-sulphate

The relatively low levels of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide determined in the urine of volunteer 1 may have been due to a low level of the enzyme necessary for metabolism of coumarin to 7-hydroxycoumarin (see section 3 2 1) However, the low levels of 7-hydroxycoumarin (free and conjugated) observed in the urine of volunteer 1 may have been related to the fact that she was undergoing hormone replacement treatment (HRT) Perhaps, coumarin metabolism or the excretion of its metabolites was being impaired by the action of the HRT Levels in the volunteers plasma were however, (see Table 4 1 3 1) almost twice as high as each of the other volunteers This may also have been due to the HRT The volunteer with the lowest plasma levels of 7-hydroxycoumarin-glucuronide had the highest levels present in their urine i e volunteer 3 A comparison of blood levels to urine levels, of 7-hydroxycoumarin/7-hydroxycoumarin-glucuronide, may give a greater insight into coumarin metabolism and drug disposition (see section 4 3)

4 1 3 3 Study 2 Analysis of serum samples from volunteers who had been treated with 7-hydroxycoumarin

Serum samples were taken from each of three volunteers who had been treated with 7-hydroxycoumarin Two separate patients were treated orally with 4 0 g of 7-hydroxycoumarin and 7 0 g was administered to another patient These patients were undergoing treatment with 7-hydroxycoumarin as part of a study into the metabolism of 7-hydroxycoumarin in patients with advanced malignancies (see section 4 3) Blood samples were removed at specific time intervals on each of two different days [Day 1 and Day 15] (Table 4 1 3 4) and allowed to clot The serum was removed and stored at -80 °C until required Samples were prepared as outlined in section 2 2 2 2 Table

4 1 3 4 are the results for 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide concentrations in the serum from two of the patients' on day 1 and day 15 of the trial

Sample Time	Patient 1 Day 1		Day 15		Patient 2 Day 1		Day 15	
	Concentrations of 7OHCG	7-OHC	Concentrations of 7OHCG	7-OHC	Concentrations of 7OHCG	7-OHC	Concentrations of 7OHCG	7-OHC
min	µg/ml		µg/ml		µg/ml		µg/ml	
0	0	0	ca 0 5	ca 0 1	0	0	ca 0 5	0
5	0	0	1 1	ca. 0 1	0	0	ca 0 5	0
15	0	0	1 2	ca. 0 1	0	0	ca 0 5	0
30	1 1	ca 0 1	1 1	ca. 0 1	0 6	ca 0 1	1 1	0
45	2 5	ca 0 1	5 4	ca. 0 1	3 6	ca. 0 1	5 0	ca 0 1
60	4 9	ca 0 2	14 1	ca 0 2	6 7	ca 0 1	16 5	ca. 0 2
120	32 1	ca 0 7	36 9	0 6	52 6	ca 0 5	48 7	ca. 0 3
240	133 9	1 2	125 3	1 3	148 4	ca. 0 8	162 0	ca. 0 6
480	64 1	ca 0 7	66 2	1 0	76 1	ca. 0 4	72 7	ca. 0 3
1440	1 4	ca 0 1	1 1	ca 0 1	5 5	ca. 0 1	1 8	ca 0 1

Table 4 1 3 4 HPLC results for the determination of 7-hydroxycoumarin (7-OHC), 7-hydroxycoumarin-glucuronide (7-OHCG) in serum after the oral administration of 4 0 g (patient 1) and 7 0 g (patient 2) of 7-hydroxycoumarin The concentrations were calculated from the standard curve of the mean absorbance ratio versus the mean concentrations calculated for each of the analytes ca. - approximately- i e levels of drug were observed but their exact quantification was not possible as their absorbance values did not lie within the limits of the linear detection range

Approximately 95% - 99 % of the 7-hydroxycoumarin detected in the serum is in the glucuronide form with the remainder circulating as free 7-hydroxycoumarin (Table 4 1 3 4) Other metabolites were not determined Over time the levels of 7-hydroxycoumarin increase as the drug is released into the circulation The maximum levels of 7-hydroxycoumarin, in the serum of each of these patients, was observed at 4 h At 24 h the levels of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide returned, almost, to the levels before administration of the drug It is assumed that this is due to its excretion in the urine Figure 4 1 3 3 shows an overlay of two

chromatograms for time 0 on day 15 and 2 h after administration of the drug for the third volunteer

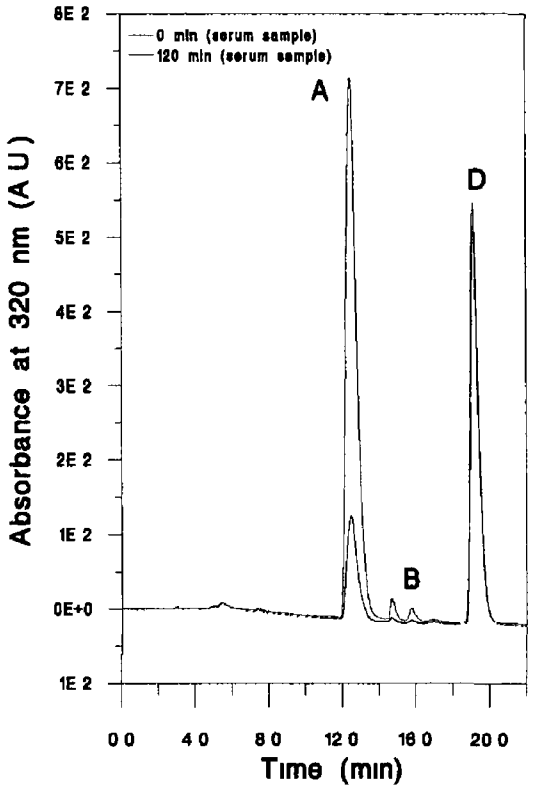

Figure 4 1 3 3 Overlay of two chromatograms of serum samples at 0 h [black] and 2 h [red] after the oral administration of 4 g of 7-hydroxycoumarin to patient 3 on Day 15 It shows the separation of (A) 7-hydroxycoumarin-glucuronide, (B) 7-hydroxycoumarin and (D) 4-hydroxycoumarin at the different times The samples were separated by reverse-phase HPLC on a C18 column with gradient elution and detection at 320 nm.

The levels of 7- hydroxycoumarin (free or conjugated) determined or the amount remaining in circulation observed at day 15 were different for each of the patients studied These samples were also analysed by the method of Egan and O'Kennedy (1992) for total 7-hydroxycoumarin content m serum The total 7-hydroxycoumarin (μg/ml) content in the serum was also determined from the results obtained after the analysis with the gradient elution method (see section 2 2 2 2 and 2 2 3 2) Table

4 1 3 5 gives the comparison of the results determined by both HPLC methods i e the method of Egan and O'Kennedy and the method described (see section 2 2 2 2 and 2 2 3 2) % of the 7-hydroxycoumarin present in the serum was present as the glucuronide form The total 7-hydroxycoumarin concentration (analysed by the method as described in section 2 2 2 2 and 2 2 3 2) was determined from the 7-hydroxycoumarin-glucuronide concentration by multiplying it by the molar ratio between the two compounds (i e 0 48 - see section 2 2 2 2 3) There is a difference of up to ± 11 % between the results

Sample Time	HPLC results Patient 1 (Day 1)		HPLC results Patient 3 (Day 15)	
	7-OHC concentration Method of Egan and O Kennedy †	7-OHC concentration HPLC method ‡	7-OHC concentration Method of Egan and O'Kennedy	7-OHC concentration HPLC method
min	µg/ml	µg/ml	µg/ml	µg/ml
0	0	0	9 1	8 1
5	0	0	8 5	7 6
15	0	0	9 0	8 1
30	1 1	0 5	8 0	7 5
45	1 8	1 2	8 0	8 0
60	3 9	2 5	9 7	7 6
120	17 3	15 4	51 3	41 3
240	61 6	64 1	93 2	86 1
480	28 8	29 3	64 3	51 4
1440	1 2	0 7	9 3	9 0

Table 4 1 3 5 A comparison of two HPLC methods for the determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in serum after the oral administration of 7-hydroxycoumarin to each of two patients The samples were analysed by the method of †Egan and O'Kennedy (1992) and by ‡ HPLC method (see section 2 2 2 2 and 2 2 3.2)

From the analysis of the serum it was clearly seen that two weeks after the initial dosage of the drug there is a constant pool of circulating 7-hydroxycoumarin-glucuronide and 7-hydroxycoumarin in the serum On day 1 no 7-hydroxycoumarin, free or conjugated,

was observed in the serum. The drug concentration time profile for the third patient after the administration of 4.0 g of 7-hydroxycoumarin on day 15 is shown in Figure 4.1.3.4.

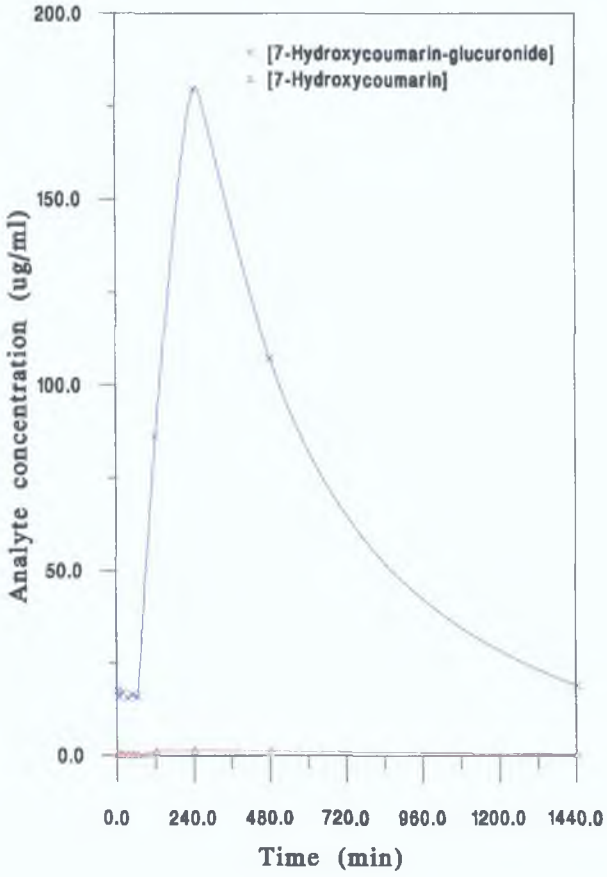

Figure 4.1.3.4. Time profile of 7-hydroxycoumarin concentration [red] and 7-hydroxycoumarin-glucuronide concentration [blue] versus time for a patient who had been administered 4 g of 7-hydroxycoumarin. The samples were taken on day 15 and analysed by reverse-phase HPLC on a C18 column with gradient elution and detection at 320 nm. Concentrations were calculated from a standard curve of mean absorbance ratio versus mean concentrations calculated.

4 2. ANALYSIS OF THE GLUCURONIDATION OF 7-HYDROXYCOUMARIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The *in vitro* metabolism of 7-hydroxycoumarin to 7-hydroxycoumarin-glucuronide was investigated in bovine liver homogenate by HPLC A metabolic reaction mixture was prepared that included a crude preparation of uridine diphosphate (UDP) glucuronyl transferase (UDPGT), 7-hydroxycoumarin and UDP-glucuronic acid (UDPGA) [see section 2 2 2 2] Over a time period of 150 min the 7-hydroxycoumarin-glucuronide produced enzymatically was determined by HPLC

Concentrations of 7-hydroxycoumarin-glucuronide produced were calculated from a plot of 7-hydroxycoumarin-glucuronide concentration versus the mean absorbance ratio (n=4) The absorbance ratio is calculated from 7-hydroxycoumarin-glucuronide absorbance/4-hydroxycoumarin absorbance The method allowed both the determination of 7-hydroxycoumarin as well as 7-hydroxycoumarin-glucuronide The identity of the compound produced was confirmed by photo-diode array spectral analysis A plot of time versus 7-hydroxycoumarin-glucuronide produced indicates that the metabolism is linear for the first 90 min and reached a plateau at 150 min The rate of reaction in the first 90 min was 2 96 ± 0 06 (R S D 1 7%, n=3) nmol of 7-hydroxycoumarin-glucuronide produced per minute per milligram of protein After 150 min 0 34 ± 0 01 mM (R S D 1 4%) of 7-hydoxycoumarin-glucuronide was produced, from 0 77 mM 7-hydroxycoumarin introduced into the reaction mixture 58 0 % ± 5 3 % (or 0 44 ± 0 02 mM) of the 7-hydroxycoumarin remained after the 150 min

4 2.1. HPLC separation parameters

HPLC was applied to the determination of 7-hydroxycoumarin-glucuronide concentration after the *in vitro* metabolism of 7-hydroxycoumarin by UDPGT The HPLC separation method details are described in section 2 2 3 2 The HPLC method had a limit of quantitation, for 7-hydroxycoumarin-glucuronide, of 0 5 µg/ml (1 47 µM) The linear range was from 0 - 100 µg/ml (0 - 295 7 µM) To prevent the endogenous β-

glucuronidase, present in the liver homogenate causing the deconjugation of 7-hydroxycoumarin-glucuronide, the standards were prepared in the reaction mixture which containing the liver homogenate denatured with trichloroacetic acid Table 4 2 1 1 gives the mean peak absorbance ratios for the 7-hydroxycoumarin-glucuronide standards and their percentage relative standard deviations (n=4)

7-hydroxycoumarin-glucuronide concentration (μM)	Mean peak absorbance ratio (n=4)	% Relative standard deviation
0	0	0
1 47	6E-3 ± 2E-4	1 3
2 96	0 01 ± 2E-4	3 0
5 92	0 02 ± 1 5E-3	8 3
14 79	0 05 ± 1 5E-3	3 2
29 58	0 09 ± 3 0E-3	3 2
59 15	0 19 ± 6 9 E-3	3 7
147 89	0 46 ± 9 9E-3	2 2
236 62	0 73 ± 40 7E-3	5 6
295 77	0 88 ± 59 0 E-3	6 7

Table 4 2 1 1 7-hydroxycoumarin-glucuronide standard concentrations (μM) and their respective mean absorbance ratios ± standard deviation (n=4), and their percentage relative deviations 7-hydroxycoumarin-glucuronide standards (n=4) were prepared in the incubation mixture, (see section 2 2 2 2) The samples were analysed by reverse-phase HPLC on a C18 column with gradient elution and detection at 320 nm The absorbance ratio was calculated from (absorbance 7-hydroxycoumarin-glucuronide / absorbance internal standard)

4 2 2 Glucuronidation of 7-hydroxycoumarin by UDPGT

7-hydroxycoumarin-glucuronide was monitored over time, as it was produced enzymatically as the *in vitro* metabolite of 7-hydroxycoumarin The increase of 7-hydroxycoumarin-glucuronide content could be observed visually (Figure 4 2 2 1) in each of the chromatograms, resulting after HPLC analysis of the samples, taken at the different times The concentration of 7-hydroxycoumarin-glucuronide produced was determined from the plot of mean peak absorbance ratio for 7-hydroxycoumarin-glucuronide versus 7-hydroxycoumarin-glucuronide concentration (Table 4 2 1 1)

Table 4 2 2 1 are the results for the mean 7-hydroxycoumarin-glucuronide concentration produced (n=3) over time (min)

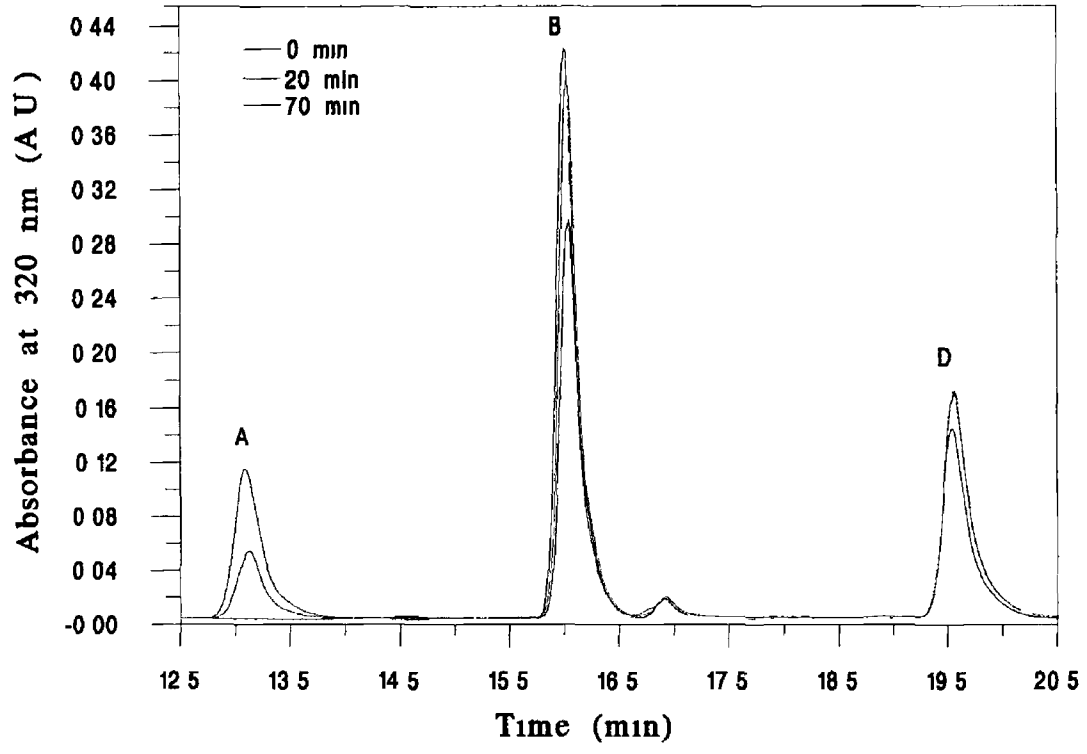

Figure 4 2 2 1 Overlay of three chromatograms, (time (min) versus absorbance at 320 nm (absorbance units) from 12 5 min to 20 5 min, of samples taken at 0 min [black], 20 min [red], and 70 min [blue], respectively, showing the decrease in (B) 7-hydroxycoumarin absorbance, and the increase of (A) 7-hydroxycoumarin-glucuronide absorbance over time. (D) is the internal standard, 4-hydroxycoumarin The samples were analysed by reverse-phase HPLC on a C18 column with gradient elution and detection at 320 nm as outlined in section 2 2 2 2 and 2 2 3 2

After 90 min the metabolic rate decreased and the level of 7-hydroxycoumarin-glucuronide no longer increased linearly Figure 4 2 2 1 shows a plot of 7-hydroxycoumarin-glucuronide produced (μM) for each of the incubation solutions analysed versus time (min) It was also possible to monitor the decrease in the 7-hydroxycoumarin content as it was consumed by the enzymatic process It was possible to observe the 7-hydroxycoumarin content decrease over time (Figure 4 2 2 1) The %

decrease in 7-hydroxycoumarin content (i e 58 %) was calculated by comparing the peak absorbance ratios for 7-hydroxycoumarin absorbance / 4-hydroxycoumarin absorbance at 0 min and at 150 min In the absence of 7-hydroxycoumarin, liver homogenate containing the UDPGT, or UDPGA, no 7-hydroxycoumarin-glucuronide was produced Using the same metabolic mixture, another similar metabolic assay study was carried out, with capillary electrophoresis (CE) as the mode of separation of 7-hydroxycoumarin-glucuronide from the reaction components, with UV detection at 320 nm (see section 2 3 2 4 and 3 2 4)

Sampling Time	**Mean 7-hydroxycoumarin-glucuronide concentration ± Standard Deviation**	**% Relative standard deviation**
min	µM	
0	0	0
10	47 1 ± 0 7	1 6
20	92 6 ± 5 5	5 9
30	122 4 ± 3 1	2 5
40	153 8 ± 3 1	2 0
50	177 8 ± 3 9	2 2
60	200 5 ± 5 5	2 7
70	233 1 ± 12 4	5 3
85	262 3 ± 4 7	1 7
90	280 4 ± 3 8	1 4
105	287 2 ± 3 0	1 1
120	302 6 ± 10 6	3 5
135	331 3 ± 0 6	2 0
150	336 7 ± 5 0	1 5

Table 4 2 2 1 Mean concentrations of 7-hydroxycoumarin-glucuronide (± Standard Deviation) over time produced by bovine liver UDPGT and the % relative standard deviation (n=3). The samples were analysed by reverse-phase HPLC on a C18 column with gradient elution and detection at 320 nm Concentrations were calculated from a standard curve of 7-hydroxycoumarin-glucuronide, prepared in the incubation mixture, versus absorbance ratio as described in section 2 2 2.2

There was no statistical difference, for the determination of 7-hydroxycoumarin-glucuronide concentration and enzyme activity, between the CE method and the HPLC method. The rate of reaction from the CE study was 3.09 ± 0.13 μM (n=3) 7-hydroxycoumarin produced per min per mg of protein as compared to 2.96 ± 0.06 μM for the HPLC method.

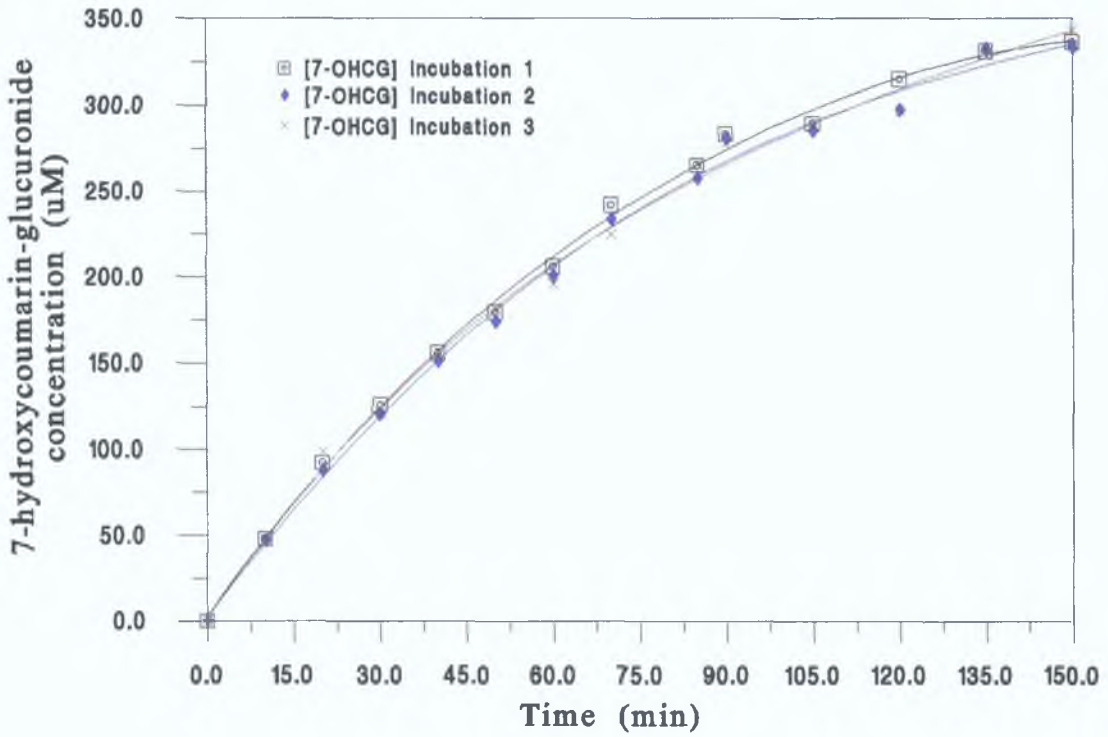

Figure 4.2.2.2. Metabolism of 7-hydroxycoumarin by uridine diphosphate glucuronyl transferase (UDPGT) with the cofactor UDP-glucuronic acid. A plot of the 7-hydroxycoumarin-glucuronide concentration (μM) (produced enzymatically) versus time (min), for each of three incubations is shown (Incubation 1 [grey]; Incubation 2 [blue]; Incubation 3 [red]). The samples were analysed by reverse-phase HPLC on a C18 column with gradient elution and detection at 320 nm. Concentrationswere calculated from a standard curve of 7-hydroxycoumarin-glucuronide, prepared in the incubation mixture, versus absorbance ratio as described in section 2.2.2.2.

The difference between the two methods, with respect to sample preparation, was that, for CE, samples were removed from the reaction mixture and analysed immediately

without any sample preparation i e without protein precipitation The application of 30 kV to the sample from the reaction mixture stopped the reaction by separating the individual components of the reaction However, it was not possible to monitor the decrease in 7-hydroxycoumarin content on CE, due to interference from magnesium chloride Magnesium chloride was found to co-migrate with the 7-hydroxycoumarin

High-performance liquid chromatography was found to be an excellent analytical technique for the determination of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide over a range of applications The method, developed and described above, was both accurate and precise in the determination of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in plasma, serum and urine It was also shown to be applicable to the investigation of the *in vitro* metabolism of 7-hydroxycoumarin to 7-hydroxycoumarin-glucuronide

The advantages of this method over other HPLC methods (e g Moran *et al*, 1987, and Egan and O'Kennedy, 1992) for the determination of coumarin and 7-hydroxycoumarin was that there was no deconjugation step, thereby reducing sample preparation time There was no extraction, no evaporation and no reconstitution steps, thus, the sample preparation time was significantly decreased There is less organic waste and the assay is less costly than the method of Egan and O'Kennedy (1992) as there was no need for expensive HPLC grade organic solvents used in the solvent extraction step (i e diethyl ether) or for β-glucuronidase The tubes used did not need to be solvent resistant Sample volumes were reduced which causes less inconvenience to volunteers or patients alike The limit of detection for the determination of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in serum and plasma was improved by a factor of 10 The analysis time of the method developed and described above was longer, 16 min for the method of Egan and O'Kennedy (1992) as compared to 30 min (see section 2 2 3 2) The above advantages outweigh this minor disadvantage The analysis and data processing was computer controlled and the samples were analysed via an autosampler (see Figure 4 0 1) Thus, time in this case does not cause any major disadvantages as analysis takes place overnight with the automated injection system and data processing

4 3. A STUDY INTO THE METABOLISM OF 7-HYDROXYCOUMARIN IN PATIENTS WITH ADVANCED MALIGNANCIES

Acknowledgement

This work was carried out in conjunction with M Ernest Marshall and Katherine Kervin of the Comprehensive Cancer Center of Alabama University at Birmingham, Birmingham, Alabama. Blood samples were taken from each of 59 patients, each with advanced malignancies, and the serum samples were forwarded from Birmingham to Dublin on dry ice for analysis

Before any drug can produce its desired action, it must first pass through several processes, all of which will have an effect on its final pharmacological profile (Bowman and Rand, 1980) A general scheme for the profile of an orally administered drug, e g 7-hydroxycoumarin, is outlined in Figure 4 3 1 The various processes involved are divided into three phases In the *pharmaceutical phase*, the formulation of an agent is changed, from the stable production form, in which it is given, to the form by which it is absorbed The *pharmacokinetic phase* encompasses the subsequent steps of absorption, distribution, metabolism and excretion The pharmaceutical and pharmacokinetic phases describe what happen to a drug This is also called drug disposition

The pharmacological effect, or what the drug does to the body, is evident in the *pharmacodynamic phase*, where the drug, in some form determined by the drug disposition, interacts with a specific system to produce the complete, therapeutic and toxic effects observed These effects are, therefore, a product of the interaction of all elements of each phase, and any variation in one may ultimately leads to a change in the whole pharmacological profile of the drug The profile of a pharmaceutical agent is strictly unique to an individual, since it represents a product of biochemical variation

The section below deals with the drug disposition of 7-hydroxycoumarin after the oral administration of a range of doses to patients with advanced malignancies with reference to distribution of the drug in free and conjugated forms into serum

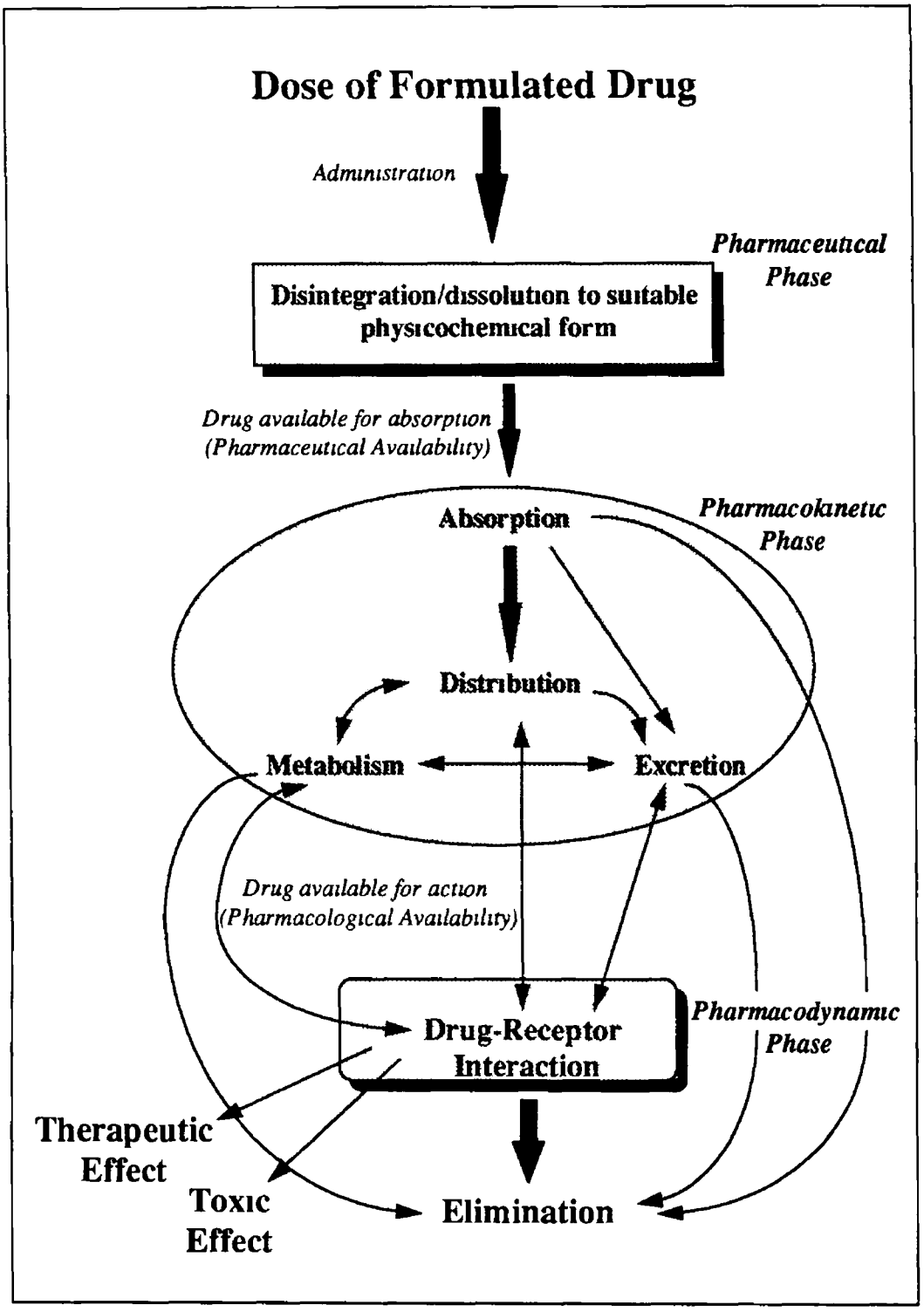

Figure 4 3 1 A flow diagram of the processes which contribute to the pharmacological profile of an orally administered drug This process is normally divided into three phases The pharmaceutical phase describes the processes taking place from the administration of an agent to release of the substance into the body fluids The pharmacokinetic phase describes what the body does to the drug and the resultant effect on the body is encompassed in the pharmacodynamic phase (Taken from Bowman and Rand, 1980, and O'Connor, 1995)

4.3.1. Objectives of the study

Coumarin and 7-hydroxycoumarin are under investigation for the treatment of certain malignancies or for the chemoprevention of malignancy (Marshall *et al*, 1994, Marshall *et al*, 1994 personal communication) 7-hydroxycoumarin was given to a large number of cancer patients over a wide range of doses with no significant toxicity While much is known of the basic pharmacology of coumarin and 7-hydroxycoumarin there is insufficient data on serum levels and glucuronidation at the high dose levels used in clinical trials Furthermore, much of the pharmacological work was performed in non-human mammalian species where the metabolism may differ from that in humans (Ritschel *et al*, 1977, 1981a, 1981b, 1983, 1984, 1988, 1992a, and 1992b, Sharifi *et al*, 1993b) Therefore the purpose of the study was to monitor serum levels of total 7-hydroxycoumarin (free and/or conjugated) levels over 24 hours following a single oral dose on each of two different days, 14 days apart

4.3 2 Phase I trial of 7-hydroxycoumarin

On the basis that coumarin may be a prodrug for 7-hydroxycoumarin (Ritschel 1992a, Sharifi *et al*, 1993b), as it is quickly metabolised to 7-hydroxycoumarin in man, 7-hydroxycoumarin was investigated for its clinical efficacy for the treatment of advanced malignancy It was also unknown whether 7-hydroxycoumarin would have less toxic side effects than coumarin as it lacks the strong aromatic properties of coumarin 7-hydroxycoumarin, the major human metabolite of coumarin, was formulated into a pharmaceutical preparation (100 mg tablets) This preparation was subjected to a phase I trial among patients with advanced malignancies which were refactory to "standard" therapies or for which no "standard" effective therapies existed Patients were treated with 7-hydroxycoumarin as a single daily oral schedule at doses 100 mg - 7000 mg (see section 2 2 2 1 and Table 2 2) The study was halted at 7000 mg due to practical reasons The patients at the highest doses were taking 70 tablets as a single daily dose The timing of the blood sampling was chosen to allow the study to be carried out on an outpatient basis Samples were taken for the first 8 hours and at 24 hours (see Tables 4 3 4 1 A-G) and analysed for total 7-hydroxycoumarin content (free and conjugated)

4 3.3 Patient selection criteria

Patients had to fulfil the following criteria to be eligible for the trial (taken from Marshall *et al* , personal communication, 1994)

1 Patients must have a histologically or cytologically confirmed diagnosis of cancer which is metastatic or unresectable

2 Patients must have bidimensionally measurable or evaluable disease which is refactory to standard therapies or for which no standard effective therapy exists

3 Patients may have received any prior therapy for their disease but must be at least 4 weeks remote from the last treatment (6 weeks if a nitrosourea) and have recovered from any toxicities associated with such therapy

4 Patients may not receive concomitant chemotherapy, immunotherapy, radiation therapy or hormonal therapy (except oral contraceptives) while on this study

5 Patients must have an ECOG performance status of 0-2

6 Patients must have adequate baseline organ functions (measured within 14 days of beginning treatment) defined as WBC ≥ 3,000/ mm^3, platelets ≥ 100,000/ mm^3, hemoglobin ≥ 10 0, normal bilirubin, 1 5 X upper limit of normal ≥ SGOT, 2 0 ≥ serum creatinine or a creatinine clearance (measured or estimated) ≥ 60 ml/min

7 Patients must be at least 3 months remote from myocardial infarction and with no residual angina or congestive heart failure Patients with angina pectoris are not eligible Patients with poorly controlled cardiac arrythmias are not eligible

8 Pregnant or lactating women are not eligible for this study Men and women of reproductive age and capacity must use an effective form of contraception

9 Patients must be informed of the investigational nature of the study and must give written informed consent to participate

4.3.4 The determination of total 7-hydroxycoumarin in serum samples

The method of Egan and O'Kennedy (1992), was developed for the determination of free and total 7-hydroxycoumarin in urine and plasma The method was then adapted and applied to the determination of total 7-hydroxycoumarin in serum samples taken from patients who had been treated with a single oral dose of 7-hydroxycoumarin

Blood samples were taken at specific times and analysed as outlined in section 2 2 2 2 and 2 3 2 1

4 3 4 1 Total 7-hydroxycoumarin concentrations

Figure 4 3 4 1 1 shows a chromatogram of 7-hydroxycoumarin standards and the internal standard spiked in serum, treated with β-glucuronidase, extracted, reconstituted and analysed by HPLC as described in section 2 2 2 2 and 2 2 3 1

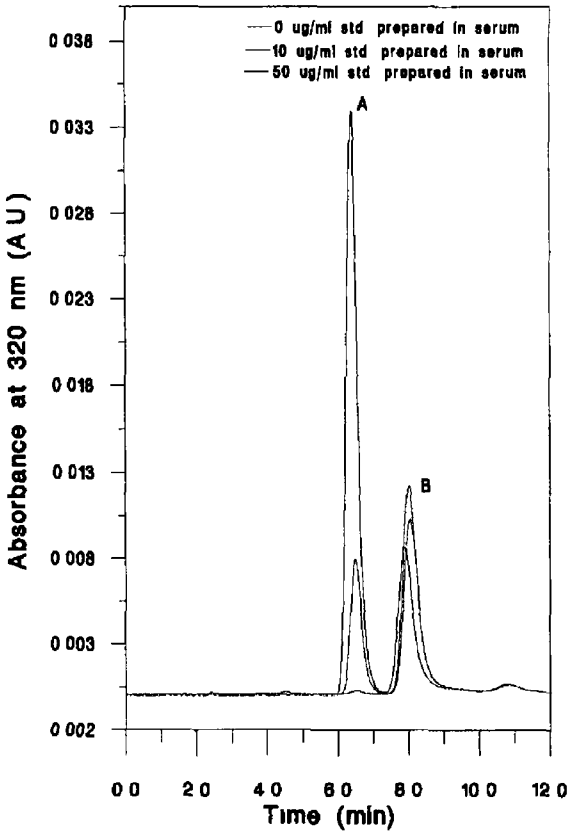

Figure 4 3 4 1 1 Chromatogram showing the overlay of the separation of (A) 7-hydroxycoumarin standards [0 μg/ml (red), 10 μg/ml (blue) and 50 μg/ml (black)] and the internal standard (B) 7-amino-4-methyl-coumarin prepared in serum, extracted and analysed by the method of Egan and O'Kennedy, as described in section 2 2 2 2 The samples were separated by reverse-phase HPLC on a C18 column under isocratic conditions (see section 4 2 3 1) with detection at 320 nm.

A standard curve of 7-hydroxycoumarin versus peak absorbance ratio was prepared as outlined under section 2 2 2 2 Figure 4 3 4 1 2 shows a typical standard curve plot obtained The equation of the line was Y = 1 26 E-2 + 4 34 E-2, R^2 = 0 99 The standard curves were prepared daily with every set of analysis of 'unknowns' The regression coefficient (R^2) was typically at least 0 99 Table 4 3 4 1 (A-G) are the results obtained for the study The study consisted of 59 patients and the range of doses was between 100 mg and 7000 mg The tables give the total 7-hydroxycoumarin (free and conjugated) concentrations, determined by HPLC, in serum from each of the patients at the different sampling times and the related dose administered

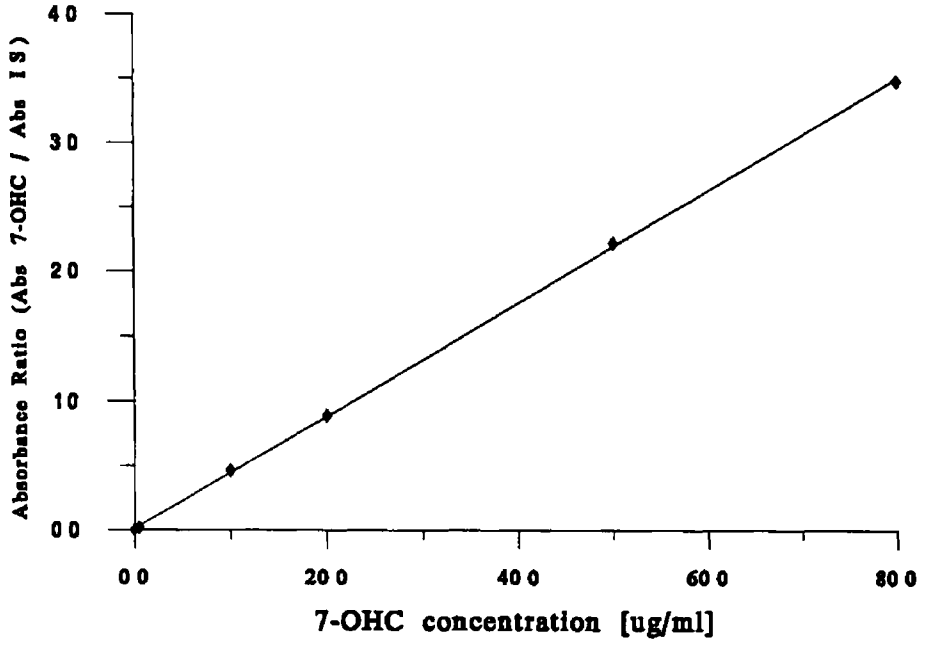

Figure 4 3 4 1 2 A standard curve plot of 7-hydroxycoumarin concentration versus peak absorbance ratio The standards were prepared in serum and treated as outlined in section 2 2 2 2 They were then analysed by HPLC as outlined in section 2 3 2 1 The peak absorbance ratio was calculated from the peak absorbances of the internal standard and the 7-hydroxycoumarin standard. Standard curves were prepared daily The equation of the line is Y = 0 01 + 0 04 X R^2 = 0 99

Time	Day 1	Day 15	Day 1	Day 15	Day 1	Day 15
min	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
	P1 100 M 56 RCC		P2 100 F 60 CAR		P3 100 M 50 RCC	
0	0	0	0	0	0	0
5	0	0	0	0	0	0
15		0	0	0	0	0
30	0	0	0	0	0	0
45	0	0 5	0	0	0	0
60	1 8	1 6	1 8	0	0	
120	0 9	1	1 3	0	0	0
240	0	0 9	0 5	1 6	0	0 6
480	0	0 6	0	0 5	0	0 9
1440	0	0	0	0	0	0
	P4 300 F 50 RCC		P5 300 F 60 RCC		P6 300 M 48 NSL	
0	0			0 5	0	0
5	0			0 5	0	0
15	0		0	0 5	0	0
30	0		0	0 5	0	
45	0		0	0 5	0	0
60	0		0	0 5	1 2	1 1
120	1 1		0	0 6	2 0	6 0
240	5 7		0	5 5	2 2	8 2
480	2 6		2 9	0 8	0	5 6
1440	0		0	0	0	0
	P7 500 F 71 RCC		P8 500 F 82 RCC		P9 500 M 70 RCC	
0	0	0	0		0	0 9
5	0	0	0		0	0 9
15	0	0	0		0	1 0
30	0	0	0		3 1	1 0
45	1 6	0	2 3		8 2	2 7
60	3 0	0	5 8		12 0	3 1
120	6 2	3 3	11 7		18 4	9 2
240	6 5	7 1	10 5		14 7	13 4
480	1 4	3 8	4 3		10 1	11 2
1440	0	0			0	

Table 4 3 4 1 A-G A table of the 7-hydroxycoumarin serum concentrations as determined by HPLC and the times at which samples of serum were taken The results are given as the patient number and the dose administered to the patient i e P5 300 F 60 RCC represents patient number 5 (female, 60 years old who was diagnosed with renal cell carcinoma) and she was administered 300 mg of 7-hydroxycoumarin Where there are spaces in the data indicates that there was no sample available at this time or the patient did not partake in the study i e patient 4 did not undergo the day 15 drug trial and thus, there was no serum samples to be analysed For the abbreviations used see Table 4 3 4 1 G The patients were administered the drug on two days, Day 1 and 14 days later i e Day 15 Serum samples were taken on both days and analysed for total 7-hydroxycoumarin.

P T O

Time	Day 1	Day 15	Day 1	Day 15	Day 1	Day 15
min	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
	P10 700 M68 COL		P11 700 F62 NSL		P12 700 F 65 COL	
0	0	0	0	0	0	0 6
5	0	0	0	0		0 6
15	0	0	0 6	0	0	0 6
30	0	0	1 0	0	0	1 1
45	0 9	0 5	1 1	0	0	1 5
60	3 5	1 6	1 3	1 1	0	2 6
120	9 2	6 6	3 6	6 0	1 3	4 2
240	6 4		8 6	8 2		2 4
480	2 0	2 3	2 4	5 6	5 1	2 2
1440	1 0	0		0		0
	P13 900 M 52 BLD		P14 900 F 62 COL		P15 900 F 27 SAR	
0	0	0	0	0	0	
5	0	0	0	0	0	
15	0	0	0	0	0	
30	0	0	0	0	0	
45	0	0 6	0	0	1 3	
60	0 7	1 4	0	0 7		
120	1 8	6 0	3 8	9 9	2 0	
240	11 0	7 0	9 6	6 9	9 7	
480	4 4	3 7	6 0	6 7	3 3	
1440	0	0	0	0	0	
	P16 1200 M 54 COL		P17 1200 F 66 NSL		P18 1200 F 38 COL	
0	0	0	0 7	0 5	0	
5	0	0	0 5	0 5	0	
15	0	0	0 6	0 5	0	
30	0	0 7	0 6	0 9	0 5	
45	0	8 6	1 3	1 9	0 7	
60	0 7	10 7	2 4	2 3	1 0	
120	1 1	20 1	9 6	5 0	1 7	
240	19 3	9 5	25 7	26 9	3 6	
480	14 3	2 0	10 4	10 8	11 6	
1440	2 8	0	0 6	0	0 8	

Table 4 3 4 1 B

P T O

Time	Day 1	Day 15	Day 1	Day 15	Day 1	Day 15
mn	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
	P19 1500 M 53 RCC		P20 1500 F 76 NSL		P21 1500 F 47 ADR	
0	0	0	0		1 1	2 9
5	0	0	0		1 1	2 5
15	0	0	1 9		1 2	2 5
30	0	0 9	3 5		1 1	3 0
45	0	2 6	5 7		1 1	6 9
60	1 1	4 5	7 9		2 1	11 8
120	5 7	14 8	27 7		26 9	23 4
240	6 6	44 0	28 0		30 8	25 9
480		2 8	9 4		12 8	22 8
1440	5 8	0	2 6		2 6	2 6
	P22 2000 F 60 RCC		P23 2000 F 68 NSR		P24 2000 M 61 COL	
0	0		0	0	0	0
5	0		0	0	0	0
15	0		0	0	0	0
30	0		0 7	0	1 8	7 8
45	0 5		1 3	0	3 4	18 8
60	3 9		4 6	0	8 9	35 0
120	25 8		36 2	3 5	11 8	50 0
240	17 9		17 2	33 6	33 6	24 7
480	2 1		7 3	11 4	11 8	8 8
1440	0		0	0		0
	P25 2500 F 75 CAR		P26 2500 M 67 COL		P27 2500 F 47 COL	
0	0	0	0	0	0	
5	0	0	0	0	0	
15	0	0	0	0	0	
30	0	0	0	0	0	
45	0	1 6	3 5	0 5	0	
60	0	6 2	5 0	3 3	0	
120	11 4	36 2	23 6	11 9	7 6	
240	32 5	39 5	15 7	50 0	33 0	
480	25 1	11 4	10 3	16 9	7 2	
1440	0	0	2 0	0 9		

Table 4 3 4 1 C

P T O

Time	Day 1	Day 15	Day 1	Day 15	Day 1	Day 15
min	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
	P28 3000 M 58 COL		P29 3000 F 57 RCC		P30 3000 F 66 RCC	
0	1 0		0 8	2 0	0	5 3
5	1 0		0 8	4 6	0	5 0
15	1 0			5 2	0	4 6
30	2 0		0 9	6 0	0	4 9
45	3 6		1 3	6 1	1 5	6 5
60	6 3		3 0	7 0	7 4	14 8
120	19 6		8 8	10 9	30 0	33 1
240	51 2		79 5	70 8	51 5	41 6
480	10 3		60 3		36 6	25 7
1440	0 8		10 6		19 1	7 6
	P31 3500 M 65 BRC		P32 3500 F 75 CAR		P33 3500 F 71 CAR	
0	0	0 6	0	2 9	0	0 8
5	0	0 6	0	2 7	0	0 8
15	0	0 6	0	2 5	0	0 8
30	12 3	2 8	0	2 5	0	1 0
45	22 0	6 1	0 4	2 3	0	2 0
60	40 5	12 1	1 7	2 0	1 0	3 6
120	52 1	45 6	12 0	2 0	9 5	13 1
240	39 8	32 0	20 2	8 3	43 7	26 7
480	6 1	5 5	39 1	13 3	35 6	33 9
1440	0			4 7	0	2 0
	P34 4000 F 69 COL		P35 4000 M 79 CAR		P36 4000 F 38 GBL	
0	0	5 4	0	0	0	6 0
5	0	4 8	0	0		4 8
15	0	4 9	0	0	0	5 0
30	0	4 4	1 1	1 2	0	5 1
45	0	5 3	1 8	5 0	0	4 9
60	0	12 7	3 9	10 3	0	4 8
120	10 6	26 7	17 3	25 6	0	5 2
240	39 2	33 2	61 6	76 1	0	5 2
480	61 6	23 1	28 8	41 0	2 9	7 6
1440	1 1	3 2	1 2	1 8	6 6	6 3

Table 4 3 4 1 D

P T O

Time	Day 1	Day 15	Day 1	Day 15	Day 1	Day 15
mm	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
	P37 4500 M 69 COL		P38 4500 M 61 PRS		P39 4500 M 66 PAR	
0	0	0	0	9 1	0	1 0
5	0	0	0	8 5	0	0 9
15	0	0	0	9 0	0	0 8
30	0	0	0	8 0	0	0 7
45	0	0	0	7 8	3 3	0 7
60	0	0	0	9 7	7 7	0 8
120	0	0	9 5	51 3	36 0	9 8
240	0	0	116 9	93 2	66 5	59 4
480	20 6	11 3	68 0	64 3	29 2	45 1
1440	11 5	8 3	5 5	9 3	3 8	0 5
	P40 5000 F 58 RCC		P41 5000 M 46 CAR		P42 5000 F 54 RCC	
0	0	0	0	1 4	0	4 5
5	0	0	0	1 2	0	3 7
15	0	0	0	1 1	0	3 4
30	0	0	0	3 2	1 9	3 5
45	4 4	1 3	1 5	13 9	7 7	3 4
60	22 2	3 7	1 0	26 8	7 9	4 7
120	54 9	6 6	17 9	41 8	12 1	5 3
240	25 3	77 4	25 7	16 0	18 3	48 4
480	5 2	9 5	6 7	14 3	50 9	62 3
1440	0	0	0	0	1 8	9 3
	P43 5500 M 65 PRS		P44 5500 M 69 PRS			
0	1 2	1 2	0	1 8		
5	1 2	1 5	0	1 7		
15	1 1	1 0	0	1 6		
30	2 6	5 0	0	1 6		
45	7 6	11 7	0	1 8		
60	13 3	25 8	0	2 2		
120	32 9	82 0	24 8	13 1		
240	100 0	78 0	50 4	48 0		
480	47 4	31 2	12 1	11 4		
1440	1 4	1 5	2 1	3 0		

Table 4 3 4 1 E

P T O

Time	Day 1	Day 15	Day 1	Day 15	Day 1	Day 15
mn	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
	P45 6000 F 72 NSL		P46 6000 F 61 REC		P47 6000 M 61 PRS	
0	0	6 1	0	3 0	0	1 1
5	0	5 8		2 6	0	1 1
15	2 0	5 3		2 6	0	1 6
30		6 0		2 6	0	1 3
45	1 2	6 0		3 9	0	17 8
60	3 9	9 0		7 9	0 9	32 7
120	18 6	56 7		41 5	10 6	67 0
240	80 1	73 5	130 2	54 2	75 6	103 5
480	32 5	89 8	71 0	75 2	40 3	
1440	3 9	4 8	4 1	8 2	1 2	
	P48 6500 M 77 RCC		P49 6500 M 61 COL		P50 6500 M 64 COL	
0	0	3 4	0	2 4	0	2 1
5	0	3 0	0	2 1	0	2 7
15	0	3 2	0	2 2	0	1 8
30	0	3 1	0	1 8	0 8	2 0
45	0	3 1	0 7	1 9	1 1	1 9
60	0	3 1	1 0	2 2	2 7	1 9
120	6 2	11 7	7 3	3 8	13 7	14 3
240	28 1	21 0	77 5	23 3	41 8	40 8
480	49 4	56 8	64 0	112 8	19 7	19 4
1440	18 6	17 6	3 1	12 4	3 0	4 4
	P51 6500 M 57 PRS		P52 6500 M 45 COL		P53 6500 M 75 PRS	
0	0	0	0	6 2	0	0 7
5	0	0	0	5 9	0	0 6
15	0	0	0	5 7	0	0 5
30	0	0	0 7	9 0	0	0 7
45	0	0 7	0 8	8 4	0 6	0 8
60	11 3	3 2	1 3	7 1	1 1	1 4
120	52 2	38 2	12 9	27 0	10 0	10 0
240	50 9	74 5	73 3	70 8	26 4	36 5
480	22 4	13 8	28 3	31 0	23 5	37 7
1440		0 9	1 9	3 6	8 9	11 7

Table 4 3 4 1 F

P T O

Time	Day 1	Day 15	Day 1	Day 15	Day 1	Day 15
min	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
	P54 7000 F 70 HEP		P55 7000 M 67 PRS		P56 7000 F 37 HEP	
0	0	32 0	0	2 9	0	1 8
5	0	28 4	0	2 9	0	1 5
15	0	27 2	0	2 6	0	1 3
30	0	22 9	0	2 6	0	1 4
45	0	21 1	0	2 6	0 7	1 9
60	0	18 9	0	3 1	1 0	2 9
120	0 7	16 3	9 5	53 7	3 0	8 3
240	29 9	61 3	131 2	144 8	10 9	15 3
480	105 2	142 3	87 8	69 4	16 2	14 2
1440	4 7	1 8	3 3	4 6	15 4	18 6
	P57 7000 M 64 PRS		P58 7000 F 60 SCL		P59 7000 M 65 RCC	
0	0 6		0	0 6	0	2 3
5	0 6		0	0 6	0	1 8
15	0 5		0	0 5	0	1 6
30	0 7		0 7	0 8	0	1 6
45	0 8		2 0	2 2	0 7	1 8
60	1 4		3 9	6 9	0 9	2 2
120	10 0		22 1	15 3	43 2	63 9
240	36 5		64 0	50 3	94 8	108 6
480	37 8		31 4	26 4	34 8	39 2
1440	11 7		3 6	2 4	3 1	7 5

Table 4 3 4 1 G

The abbreviations used, for the cancer diagnosis for each patient in the table, are as follows RCC - renal cell carcinoma, NSL - non-small cell lung, PRS - prostate, REC - rectal, HEP - hepatoma, SCL - small cell lung, CAR - carcinoid, COL - colon, GBL - gall bladder, BRC - bronchial carcinoma, NRS - neurosarcoma, ADR - adrenal, SAR - sarcoma, BLD - bladder

4 3 4 2 The analysis of the data

On direct observation of the results it is clearly seen that there is a large interindividual variability in the levels of total 7-hydroxycoumarin circulating in the serum of patients after the administration of the drug The time at maximal concentration varies from patient to patient A large proportion of the patients have a maximal concentration of 7-hydroxycoumarin at 4 h but some also have peak maxima at 2 h, and at 8 h The profile for drug level in the blood for a 'typical' patient is shown in Figure 4 3 4 2 1

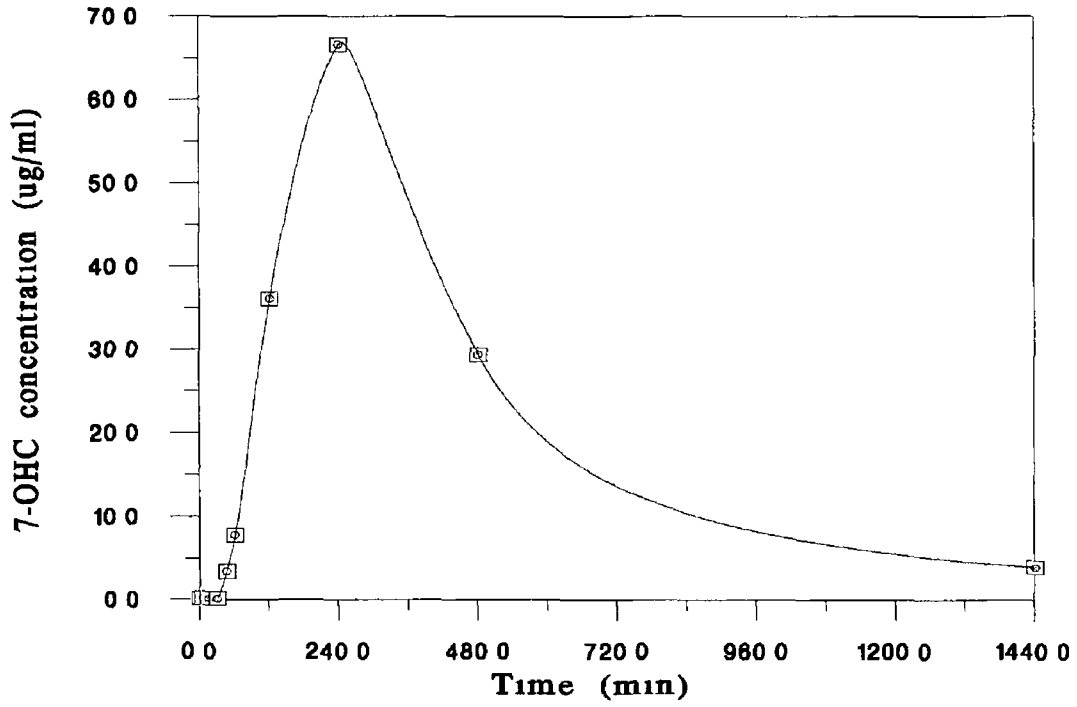

Figure 4 3 4 2 1 Plot of time versus total 7-hydroxycoumarin concentration for a patient who had been treated with 4 5 g of 7-hydroxycoumarin (P39 4500 -see Table 4 3 4 1 E) It is clearly seen that the peak maxima is at 4 h and after 24 h the level of drug in the serum has decreased significantly The samples were prepared as described in section 2 2 2 2 and analysed by reverse-phase HPLC on a C18 column under isocratic conditions with detection at 320 nm The concentrations were calculated from a plot of absorbance ratio versus 7-hydroxycoumarin concentration (see Figure 4 3 4 1 2)

Unfortunately serum samples for some of the patients were not available and thus, it was sometimes impossible to determine peak maxima times and maximum 7-hydroxycoumarin concentrations A single oral dose of the drug was administered to each of the patients on each of two days On day 1 and day 15 samples of blood were taken before and after administration of 7-hydroxycoumarin It was possible in some patients to observe a circulating 'pool' of the drug 14 days after the initial administration e g P38, P54 In patients P21, P57 7-hydroxycoumarin was found in their serum samples before they were treated on Day 1 The source of the 7-hydroxycoumarin was not determined as the clinical data and history for each patient was not included with the serum samples It was not possible to access the clinical data and correlate it to the observed serum profiles, the concentrations of 7-hydroxycoumarin determined, the drug disposition and the peak maxima In order to correlate the results it was decided to determine the area under the curve from a plot of time versus total 7-hydroxycoumarin concentration (Figure 4 3 4 2 2)

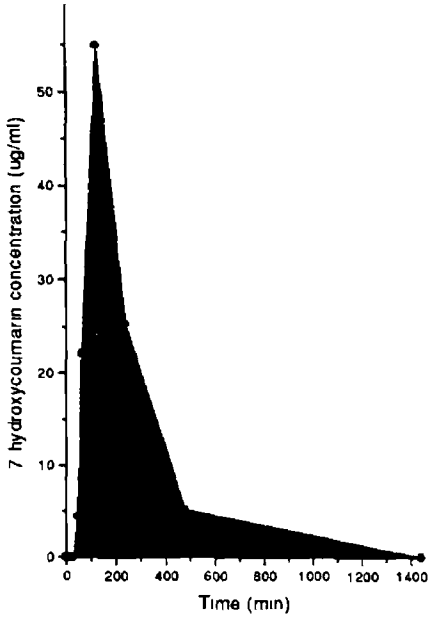

Figure 4 3 4 2 2 Plot of time versus total 7-hydroxycoumarin concentration in serum for a patient who had been orally treated with 7-hydroxycoumarin The area under the curve (shaded area) was calculated and utilised in assessing a relationship between dose administered and drug disposition The serum samples were prepared as described in section 2 2 2 2 and analysed by reverse-phase HPLC on a C18 column under isocratic conditions with detection at 320 nm The concentrations were calculated from a plot of absorbance ratio versus 7-hydroxycoumarin concentration (see Figure 4 3 4 1.2)

This would allow the determination of a relationship, if any, between the dose administered and the level of total 7-hydroxycoumarin found circulating in the serum When the area under the curve is plotted against the dose administered it is possible to see that there is a very large interindividual variability between the patients for each of the doses administered (Figure 4 3 4 2 3)

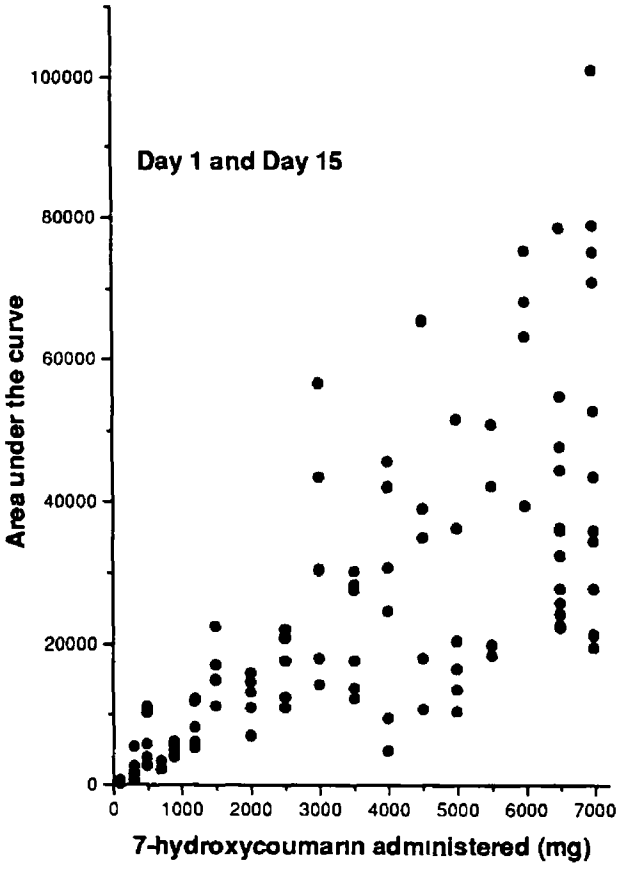

Figure 4 3 4 2 3 A plot of the area under the curve (see Figure 4 3 4 2 2) versus 7-hydroxycoumarin dose administered (mg) The plot includes the data observed for patients (with complete data sets) for analysis on both days of the study The plot shows a large variability in the amount of 7-hydroxycoumarin present in the patients serum for each dose administered and between different doses

Figure 4 3 4 2 4 is a plot of the mean area under the curve ± standard deviation about the mean for each of the doses administered (the results from the studies on both days

are combined for this figure) The plot of the mean area under the curve versus the dose of 7-hydroxycoumarin administered shows a linear relationship between the amount of drug present over time and the dose administered (Figure 4 3 4 2 4) Table 4 3 4 2 1 are the results in tabular form It is clearly seen from the results in the table that there is up to 80 % difference between some patients in the same dosage group The results were determined for complete sets of data and all incomplete data sets were ignored

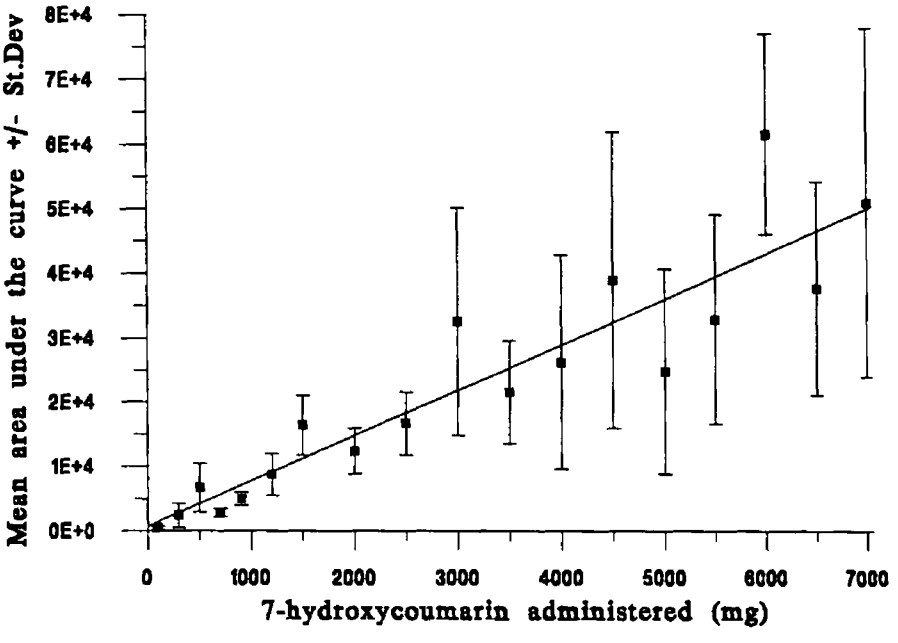

Figure 4 3 4 2 4 A plot of the mean area under the curve ± standard deviation versus the dose of 7-hydroxycoumarin administered to each patient The equation of the line is Y = 733 5 + 7 03X R^2 = 0 84 Serum samples were taken from patients treated orally with 7-hydroxycoumarin, and analysed by HPLC (see section 2 2 2 2 and 2 2 3 1) The area under the curve was determined from the profile of time versus total 7-hydroxycoumarin concentration for each patient (see Figure 4 3 4 2 2)

The results display that there is a large interindividual variability in 7-hydroxycoumarin metabolism and release into the circulation This has previously been observed for coumarin (Rautio *et al*, 1992, Iscan *et al*, 1994) and is now shown for 7-hydroxycoumarin The standard deviation about the mean area under the curve varies

from 20 % to 76 % (Table 4 3 4 2 1) again illustrating the interindividual variability in the 7-hydroxycoumarin metabolism

7-Hydroxycoumarin Administered	Mean Area Under the Curve	± Standard Deviation	% RSD	n = x
(mg)	(AU)			
100	468 3	239 3	51 0	5
300	2407 2	1835 6	76 0	5
500	6688 2	3715 0	55 5	5
700	2761 5	701 2	25 4	4
900	4932 0	981 6	20 0	5
1200	8712 4	3215 6	36 9	5
1500	16315 5	4674 8	28 6	5
2000	12327 0	3526 2	28 6	5
2500	16689 6	4919 9	29 5	5
3000	32489 8	17650 8	54 3	5
3500	21545 5	7988 4	37 1	6
4000	26163 3	16600 0	63 4	6
4500	38842 0	22987 0	59 2	6
5000	24724 0	15984 5	64 2	6
5500	32718 7	16228 0	50 0	4
6000	61423 5	15424 4	25 1	4
6500	37559 0	16549 8	44 0	12
7000	50876 8	26860 4	52 8	11

Table 4 3 4 2 1 Table of the mean area under the curve ± standard deviation, and % relative standard deviation (% RSD) and the number of data points (n = x) Serum samples were taken from patients treated orally with 7-hydroxycoumarin, and analysed by HPLC (see section 2 2 2 2 and 2 2 3 1) The area under the curve was determined from the profile of time versus total 7-hydroxycoumarin concentration for each patient (see Figure 4 3 4 2 2) The mean area under the curve was determined for n=4 - n=12, for each of the doses of 7-hydroxycoumarin administered (100 mg - 7000 mg)

Hansen and Stentoft (1995) and Herman *et al*, (1994) reported on interindividual variability in the rates of glucuronidation Hansen and Stentoft (1995) reported up to a seven fold difference in uridine diphosphate glucuronyl transferase activity in human livers for the glucuronidation of benzazepine They also reported an age-related

decrease in activity There was no age related differences in the glucuronidation of 7-hydroxycoumarin observed in this study (results not shown) Herman *et al*, (1994) reported on the metabolism of diflunisal to diflunisal sulphate and diflunisal phenolic glucuronide and diflunisal acyl glucuronide in 110 volunteers They found that the metabolism of the drug is altered by cigarette smoking and oral contraceptives The medical histories of each of the patients or their smoking status was not included It was, thus, not possible to assign the interindividual variation in the glucuronidation of 7-hydroxycoumarin to their smoking status (i e smoker or non-smoker) or if they were taking oral contraceptives

Coumarin is metabolised in humans by cytochrome P4502A6 (see section 3 2 1) However, the 'phase I' metabolism step is by-passed and the drug progresses straight to glucuronidation, referred to as 'phase II' metabolism (see section 3 2 3) The majority of work previously carried out has focused on the Phase I metabolism and the variability both between species and within species variability (see section 3 2 1) The results, shown here, also show a large scale variability in the phase II metabolism and the enzymes involved in the further metabolism of coumarin in man after the initial phase I metabolism Thus, the large interindividual variability in coumarin metabolism is not dependent solely on the phase I metabolising enzymes but also phase II metabolism

It has been proven both *in vivo* and *in vitro* that there is this variability in the cytochrome P450 enzyme activity within species and between species but in addition to this fact it must now be correlated with the fact that there is also a large variability in the secondary metabolism of coumarin i e the glucuronidation of 7-hydroxycoumarin, the release of the drug into the serum and the availability of free or conjugated drug Free drug was not determined as part of this study for practical reasons However, the serum samples were also analysed by another HPLC method (see section 4 1 3 3) and it was possible to determine both free and conjugated drug circulating in the serum 98 % of the 7-hydroxycoumarin present in the serum was present as the glucuronide form with up to only 2 % of it was circulating in the free form The exact pharmacological role of the glucuronide conjugate has not as yet been determined (a review on glucuronides is

given in section 3 2 3) Again it was not possible to access the clinical effects of the drug and the response of the patients to the treatment The clinical results have not been released However, the true maximally tolerated dose and dose-limiting toxicity were not determined in the study due to the low level of toxicity from the drug

Summary

HPLC was shown to be an excellent tool for the determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in serum, plasma, urine and for studying the *in vitro* metabolism of 7-hydroxycoumarin HPLC was also used for the determination of 7-hydroxycoumarin after the *in vitro* metabolism of coumarin (see section 3 2 2 1) The determination of 7-hydroxycoumarin in urine was determined by an enzyme-linked immunosorbent assay (ELISA) and compared to HPLC (see section 5 4 1) There was no statistical difference between the two methods All samples analysed by capillary electrophoresis were also analysed by HPLC and there was no statistical difference between either of the methods

CHAPTER 5

PRODUCTION AND CHARACTERISATION OF AN ANTI-7-HYDROXYCOUMARIN ANTIBODY

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5 2. PRODUCTION OF 7-HYDROXYCOUMARIN-PROTEIN CONJUGATES USED FOR IMMUNISATION TO PRODUCE ANTI-7-HYDROXYCOUMARIN ANTIBODIES AND FOR THE SCREENING OF THESE ANTIBODIES.

5 2.1 Synthesis and characterisation of 7-hydroxycoumarin derivatives

5 2 1 1 3-acetylamino-7-acetoxycoumarin

5 2 1 2 3-amino-7-hydroxycoumarin

5 2 1 3 Characterisation of thyroglobulin-7-hydroxycoumarin and bovine serum albumin-7-hydroxycoumarin conjugates

5 2 1 3 1 Ultraviolet (UV) spectral analysis

5 2 1 3 2 Analysis of conjugates by gel filtration - Sephadex G25 column

5 2 1 3 3 Analysis of the conjugates by HPLC

5 3. PRODUCTION AND PURIFICATION OF ANTI-7-HYDROXYCOUMARIN ANTIBODIES

5.3 1 Purification of anti-7-hydroxycoumarin antibodies from serum

5 3.2 Analysis of an anti-7-hydroxycoumarin antibody by HPLC

5.3 3 Determination of antibody activity titre

5 3 4. Cross-reactivity of the anti-7-hydroxycoumarin antibody

5 3 5. The use of the anti-7-hydroxycoumarin antibody for the determination of free and total 7-hydroxycoumarin in urine

5 3 5 1 ELISA method

5 0 INTRODUCTION

This chapter describes the production of polyclonal sera to 7-hydroxycoumarin Drugs (with a molecular weight less than 5000 Da - Catty and Raykundalia, 1988) like 7-hydroxycoumarin are too small to elicit an immune response and must be conjugated to other molecules (usually a protein) for use in antibody production Examples of proteins used for conjugation are thyroglobulin, bovine serum albumin (BSA), ovalbumin and keyhole limpet haemocyanin (KLH) These protein-drug conjugates are immunogenic and can be used for the raising of antibodies after immunisation of a host One of the protein-drug conjugates is generally used for immunisation while another conjugate is used for screening for antibody production The antiserum was produced in rabbits, by immunising with 7-hydroxycoumarin, coupled to a protein (thyroglobulin), and the resultant antibodies were purified and used in an enzyme-linked immunosorbent assay (ELISA) for the determination of free and total 7-hydroxycoumarin in urine The anti-7-hydroxycoumarin antibodies produced were also used for the development of a bispecific antibody (anti-7-hydroxycoumarin - anti-alkaline phosphatase antibody) [see section 1 4 1 1 2], in electrochemical based sensors for the determination of 7-hydroxycoumarin in urine (see section 1 4 2) and in the determination of total 7-hydroxycoumarin in serum on a commercial biosensor system - the BIAcore™ from Pharmacia Biosensor (see section 1 4 3)

5 1 ANTIBODIES

The ability to stimulate the production of specific antibodies, in a particular host, is not an inherent property of a substance An antigen is a substance capable of stimulating the immune system of an animal to produce a response specific to it - this may be antibody production, cell-mediated immunity or tolerance (Catty and Raykundalia, 1988, Roitt *et al* , 1989, Roitt, 1994, Kuby, 1994) Antigens must be 'foreign' to activate the defence mechanisms and it must be of a certain complexity to react with the different components of the immune system necessary to induce the immune response (Tijssen, 1985) Antigens may be divided into two main groups 1 Immunogens are substances

which are themselves capable of eliciting an antibody response Their antigenic determinant usually consists of a short sequence or part of the whole molecule called an epitope which 'fit' into an antigen-antibody binding site (the paratope) The epitopes of one antigen are usually different from those on another antigen Some antigens have repeated epitopes Epitopes are molecular shapes recognised by the antibodies and cells of the adaptive immune system Each antibody recognises one epitope rather than the whole antigen Even simple organisms have many different antigens 2 Another form of antigen is a hapten Haptens are substances which themselves are incapable of inducing an immune response but will do so if attached to a carrier like a protein e g BSA, thyroglobulin or KLH

"The recognition of a foreign antigen is the hallmark of the specific adaptive immune response" (Roitt *et al* , 1989) Two distinct molecules are involved in the process i e the immunoglobulins (Ig) and the T-cell antigen receptors Antibodies are a group of glycoproteins present in the serum and tissue fluid of all mammals They are produced by plasma cells which have developed from precursor B lymphocytes Contact between these B lymphocytes and a foreign antigen is required for the induction of antibody formation

5 1 1 Antibody structure and functions

The structure of an antibody is shown in Figure 5 1 1 An antibody consists of heavy and light chains which are joined by disulphide linkages A 'basic' antibody consists of two light (ca 25 kDa) and two heavy (ca 50 kDa) chains and has a relative molecular mass of 150-160 kDa The (H) heavy and (L) light chains of the antibody can be further divided into variable (V) and constant (C) regions C_H1, C_H2 and C_H3 refer to various constant domains on the heavy chain Within the variable regions there are certain areas which are highly conserved and provide a backbone for the structure There are also hyper-variable areas called complementarity determining regions (CDR) which are involved in defining the binding specificity of the antibody (Killard et al , 1995, Cahill *et al* , 1995) The constant regions (Fc) of the antibody are involved in binding to cell surface receptors The Fc region undergoes a change in conformation on antigen

binding to the Fab region The hinge region (H) allows flexibility and some degree of freedom for the two arms of the antibody molecule The antibody exposes sites, using conformational 3-D structural changes, for antigen binding The 3-D structure of IgG consists of β-pleated sheets (constant region), connecting loops (hypervariable regions), and β-sheets connected by disulphide linkages which sandwich the hydrophobic interior The carbohydrate moiety on the C_H2 region functions in stabilisation of the antibody, in inhibiting proteolytic digestion and secretion and may play a role in specifying antibody configuration There are five classes of immunoglobulins i e IgG, IgA, IgM, IgD and IgE IgG is the predominant form of antibody found in the serum

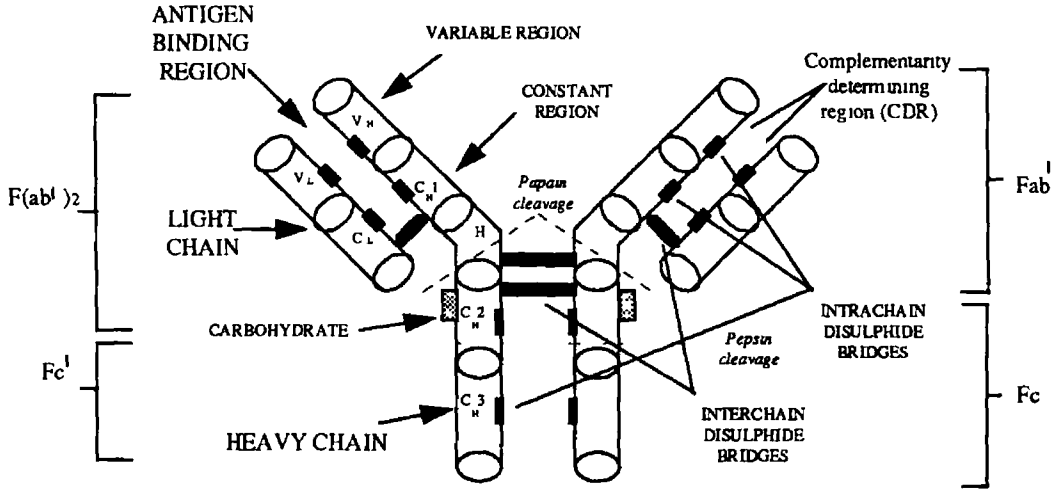


Figure 5 1 1 The basic structure of an immunoglobulin (IgG) An antibody consists of heavy (H) and light chain (L) which are joined by disulphide linkages with carbohydrate chains on the constant heavy chain (C_H2) The antibody can be further divided into variable (V) and constant (C) regions $C_H1,2,3,4$, refer to various constant functional domains There is also the hinge (H) region that allows flexibility and some degree of freedom for the two arms of the antibody molecule The antibody can be broken down into fragments by treatment with enzymes such as pepsin and papain Two antigen binding fragments (F(ab')) of the IgG, containing one antigen binding site each, and one crystallisable fragment (Fc) are produced by papain digestion of the IgG The antigen binding fragment ($F(ab')_2$) of the IgG, containing two antigen binding sites, is obtained by pepsin digestion of the IgG The heavy chain fragments remaining after pepsin digestion are called Fc' fragments The complementarity determining region (CDR) are the hyper-variable regions within the variable regions and are primarily responsible for antigen binding The structure is adapted from the structure in Killard et al , (1995)

5 1 2 Antibody-Antigen interaction

5 1 2 1 Forces that bind Antibody to Antigen

The folding of the variable chains of the light and heavy chains (see Figure 5 1 1) forms a 3-D binding spaces which are complementary, with respect to its shape and its charge distribution, to the antigenic determinant allowing it to 'fit' into the binding site The forces that hold an antigen and an antibody together may be classified under four headings (Roitt, 1980, Roitt 1994)

(a) *Electrostatic forces* are due to the attraction between two oppositely charged ionic groups on a protein side chains e g the binding of and ionised amino group (NH_3^+) and an ionised carboxyl group (COO^-) The force of attraction (F) is inversely proportional to the square of the distance between the charges (Equation 5 1 2 1 1)

Equation 5 1 2 1 1 $F \propto I/d^2$

(b) *Hydrogen bonding* These are relatively weak and reversible hydrogen bridges formed between hydrophilic groups such as -OH, $-NH_2$, and -COOH and the forces are dependent on the close approach of the two molecules carrying these groups

(c) *Hydrophobic attraction* is the interaction between hydrophobic groups to exclude water If the net surface, of the hydrophobic region, in contact with water is reduced (e g after the exclusion of water by the binding of antibody-antigen) there is a lower energy state formed between the two species, relative to no binding, forming a stable bond with a high force of attraction It has been estimated that the hydrophobic forced may contribute up to 50 % of the total strength of the antigen-antibody bond

(d) *Van der Waals Forces* This is the attraction between the external cloud electrons on the two species The force of attraction is inversely proportional to the seventh power of the distance (Equation 5 1 2 1 2)

Equation 5 1 2 1 2 $F \propto I/d^7$

5 1 2 2 Antibody Affinity

The antibody affinity is the strength of a single antibody/antigen bond, i e it is the sum of the attractive and repulsive forces between the antigen and the antibody Figure 5 1 2 2 1 shows three of the possible affinities of an antibody/antigen interaction Antibody 1 'fits' with nearly the whole of the hapten and is of high affinity Antibody 2 fits with less of the molecule and not so tightly, and has a moderate binding affinity while the low affinity antibody 3 is complementary in shape to so little of the hapten surface that its binding energy is very little above that occurring between completely unrelated proteins or other macromolecules

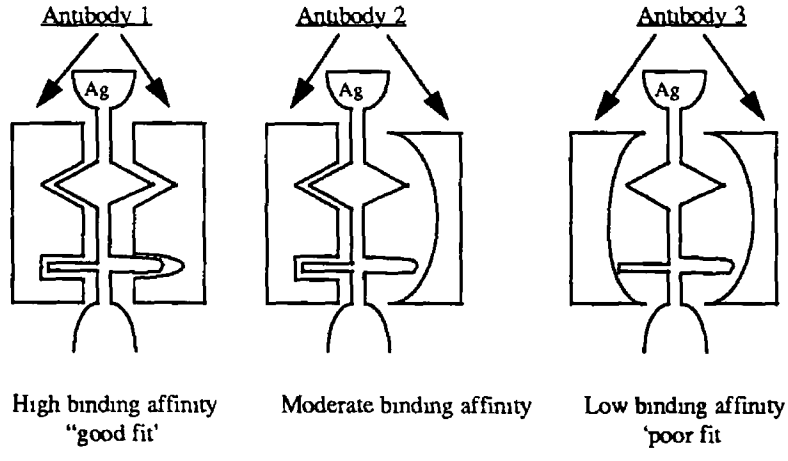


Figure 5 1 2 2 1 Schematic representation of binding of antibodies present in the same antiserum with different 'affinities' to the same antigen Antibody 1 fits with nearly the whole of the hapten and is thus of high affinity Antibody 2 fits with less of the molecule and not so tightly, and has a moderate binding affinity while the low affinity Antibody 3 is complementary in shape to so little of the hapten surface that its binding energy is very little above that occurring between completely unrelated proteins Only a portion of the antibody binding site is shown Diagram adapted from Roitt (1980)

The better the 'fit', both structurally and in charge distribution allowing the interactions detailed in section 5 1 2 1, the stronger the binding The combination of the antibody with the antigen is reversible and the complex may dissociate, depending on this binding

strength (Equation 5 1 2 2 1) Equation 5 1 2 2 1 is the equilibrium formed between antibody (Ab) and antigen (Ag) and immune complex (Ab-Ag) where K_a and K_d represent the association and dissociation constants (Tijssen, 1985)

Equation 5 1.2.2 1 $$[Ab] + [Ag] \underset{K_d}{\overset{K_a}{\rightleftharpoons}} [Ab\text{-}Ag]$$

The equilibrium constant (average affinity constant - K, units are *l/mol*) for the reaction (see Equation 5 1 2 2 1) between the antibody [Ab] and the hapten [Ag] is given in Equation 5 1 2 2 2 When the antibody and the antigen fit well together (high affinity binding - Figure 5 1 2 2 1) one gets an equilibrium that lies to the right and vice versa for low affinity binding

Equation 5 1 2 2 2 $$K = \frac{K_a}{K_d} = \frac{[Ab\text{-}Ag]}{[Ab][Ag]}$$

The affinity is dependent on the area of contact, on the approach of the two substances together and on the positioning of charged and hydrophobic groups in their respective 3-dimensional structures Antisera of multiple specificity (see section 5 1 3 2) cannot be assessed for affinity but the general characteristics of the binding reaction, e g the strength of binding, can be utilised for comparison of one antibody with another antibody This is the avidity of antibody interaction

5 1 2 3 Avidity and the bonus effect of multivalent binding

The strength of the interaction of antibody with a monovalent hapten or a single antigen determinant is called antibody affinity In most situations the avidity, i e the binding due to the interaction of the antiserum with a multivalent antigen molecule, is of greater concern (Equation 5 1 2 3 1 - Roitt, 1980) Avidity is 'how well' the antigen binds to the antibody and it is dependent on the number of binding sites on the antigen (see Figure 5 1 2 3 1) It is the performance of the antiserum (antibodies) in a particular assay (Catty and Raykundalia, 1988)

Equation 5 1 2.3 1 $$n\text{Ab} + m\text{Ag} \rightleftharpoons \text{Ab}_n\text{Ag}_m$$

Figure 5.1.2.3.1. illustrates the 'bonus' effect of antibody-antigen bonds. The binding of an antibody to two antigen molecules is greater than the sum the individual antibody links. The bond increases with increasing numbers of bridges formed between different antibodies to other epitopes.

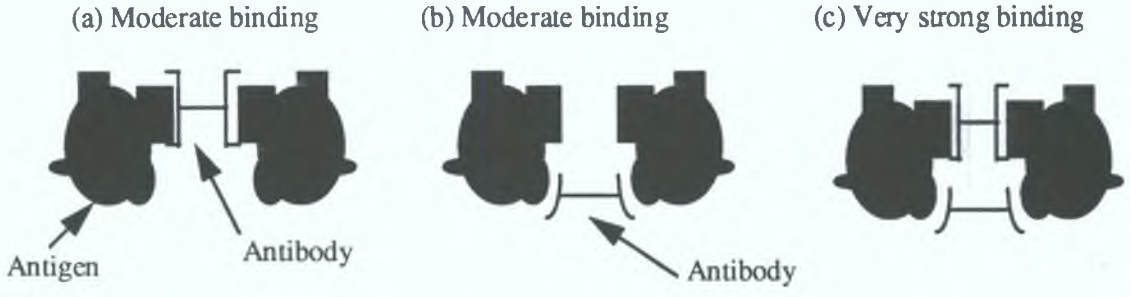


Figure 5.1.2.3.1. Schematic representation of the 'bonus' effect of multivalent attachment on binding strength. The force binding the two antigen molecules in (c) with two antibody bridges is often at least ten times greater then (a) + (b) where only single antibody bridges are formed. The effect varies with the average affinity constant (K) [see equation 5.1.2.2.2.]. Diagram taken from Roitt (1980).

The antibody-antigen reaction is reversible. If there is only one antibody binding two antigens i.e. one at each arm of the antibody, there is the possibility of separation of antibody and antigen with loss of the antigen. However, the formation of two antibody-antigen bridges will reduce the opportunity for dissociation of the antibody-antigen complex. This is illustrated in Figure 5.1.2.3.2.

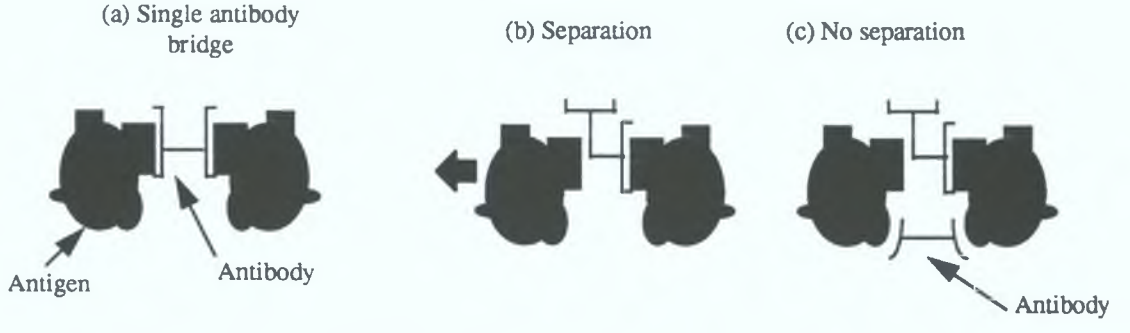


Figure 5.1.2.3.2. Schematic representation of the mechanism of the bonus effect. Each antigen-antibody complex bond is reversible and with a single antibody bridge between the two antigens (a), dissociation of either bond could result in the 'loss' of one antigen as in (b). Two antibody bridges, even when one is dissociated, prevents the 'loss' of the second antigen, holding it in place for the reform of the other antigen-antibody bond. Diagram taken from Roitt (1980).

5 1 2 4 Cross-Reactivity

An antiserum raised against a particular epitope or hapten may 'cross-react' with a partially related antigen which may have identical or similar determinants on its surface Figure 5 1 2 4 1 illustrates this effect

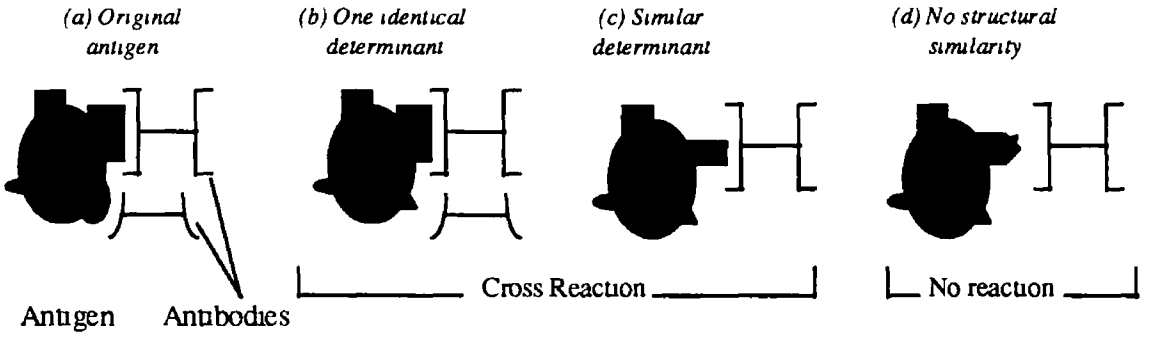


Figure 5 1 2 4 1 Schematic representation of the cross-reactivity and specificity of the antigen-antibody bond The avidity of the serum is greatest in (a) > (b) > (c) ≥ (d) so that the antiserum shows specificity Two antibodies in (a) bind to the antigen In (b) only on of the antibodies has a strong binding affinity whereas the second antibody does not have any affinity for the antigenic site In (c) the antigenic site is similar to the site in (b) and the antibody can recognise this and bind (with a lower affinity) - this antibody will show 'cross-reactivity' with the actual antibody to this site In (d) there is no structural similarities between the antigenic sites and the antibodies and no binding will occur or it will be very low Diagram taken from Roitt (1980)

The antiserum raised against the 'original antigen' will react more strongly than that of the antigen with an identical determinant which in turn reacts more strongly than the antigen with a similar determinant The weakest interaction will be for the antigen that has no structural similarity, i e there will be no significant reaction observed The specificity of the antiserum is thus determined by the cross-reactivity of the antibody-antigen (Section 5 3 4 describes the cross-reactivity of the anti-7-hydroxycoumarin antibody produced)

5 1 3 Antibody production

There is no single procedure for immunisation that guarantees an ideal antibody for a specific requirement Certain principles may be adopted which give a procedure which may be suited to a particular problem as immunogens and animals differ The essential properties of the antisera produced must be a high titre, good affinity and specificity (Catty and Raykundalia, 1988) The degree of stimulation of antigen-sensitive cells of the animals immune system will determine the affinity of the antibody There must be a high enough quantity of the immunogen present to allow an immune response Two criteria for antibody production are

1 Does the antigen need to be conjugated to a carrier molecule or is it large enough to elicit an immune response by itself ?

2 How foreign is the antigen to the host (does the antigen require assistance to elicit an immune response by the addition of adjuvant and T-Helper cells to stimulate antibody production) ?

As soon as these questions are answered it is then possible to stimulate the immune system of a host species for the production of antibodies directed against the immunogen

The steps for antibody production are as follows

A host is chosen depending on the particular application Many different animals are used as host species e g mouse, rabbit, goat, donkey, horse and human The antigen can be soluble e g a protein, or insoluble e g protein-hapten The antigen is generally prepared in adjuvant, e g Freunds adjuvant (the adjuvant increases level of antibody response by causing inflammation and attracting macrophages to the site of injection) Having chosen the host, it is immunised, generally intradermally, subcutaneously or intraperitoneally with the immunogen or protein-hapten The host will respond to the invading species and produce antibodies In order to increase the quantity of antibody response the host is 'boosted' by re-immunising Blood is taken from the host and the antibody titre (what dilution of antibody still gives reactivity against the antigen *in vitro*), specificity, affinity, stability, purity, class and isotype can be determined

Some of the advantages of rabbit and mouse antisera are now compared The applicability of rabbit antisera in precipitation reactions, and the relative ease of handling and breeding of rabbits make them ideal hosts - the immunoglobulin is easy to purify and the antibodies produced are generally of high avidity/affinity for a particular immunoassay Mice only produce a few millilitres of blood and usually require sacrifice whereas rabbits can provide up to 100 ml or more without sacrificing the animal Rabbit antisera is used for the preparation of polyclonal antibodies (see section 5 1 3 1) Mouse spleen is usually used for the preparation of monoclonal antibodies (see section 5 1 3 2)

5 1 3 1 Polyclonal antibodies

Polyclonal antiserum is the serum product of an immunised animal, usually a rabbit, sheep or goat The major advantage of polyclonal sera is its capacity to form large insoluble immune complexes with antigen, or in agglutinating cells readily so that the reactions can be seen and measured visually or photometrically The disadvantages, which may limit their exploitation in immunoassays, is the heterogeneity in specificity between batches and between animals The polyclonal sera produced is heterogenous in the classes and subclasses, in the specificity, in the titre and in the affinity of the antibodies produced In one antiserum the antibodies present will be to many different antigens, or to only a few but the antibodies produced will never be homogenous Different antibodies may compete for the same epitope with varying degrees of affinity (see section 5 1 2 2) The polyclonal antiserum produced, after immunisation, will be unique in specific antibody composition, in optimal binding conditions and in its performance in an immunoassay Affinity purification may be necessary for individual applications, e g preparation of bispecific antibodies (see section 5 1 3 4) The determination of fine structural and antigenic differences at the individual epitope level is not feasible with polyclonal antisera due to the heterogeneity and variations between batches and animals

Polyclonal antibodies can be developed quickly and relatively cheaply and do not require the amount of time or expertise required for the production of monoclonal antibodies

(see section 5 1 3 2) Antiserum levels can be determined by ELISA and qualitatively by immunoelectrophoresis When a high titre of antibody is found the polyclonal antibodies can be purified by ammonium sulphate precipitation and affinity chromatography Milligram quantities of purified antibody may be obtained from a few millilitres of serum Table 5 1 3 1 is a comparison of some of the properties of monoclonal and polyclonal antibodies (Tijssen, 1985)

Property	Monoclonal	Polyclonal
Purity of immunogen	not of prime importance	important
Time and expense	initially high	little
Useful antibody content	0 5-5 mg/ml (ascites) 5-40 µg/ml (medium)	< 1 mg/ml-few mg/ml
Irrelevant Ig	0 5-1 mg/ml	about 10 mg/ml
Physicochemical properties	individual	spectrum which changes
Cross-reactivity to copurifying immunogens	negative (-)	positive (+)
Cross-reactivity removable ?	no	mostly
Number of epitopes recognised	1	many
Affinity	homogenous	heterogenous

Table 5 1 3 1 Comparison of some the properties of monoclonal and polyclonal antibodies Table taken from Tijssen (1985)

5 1 3 2 Monoclonal antibody production

In 1975, Kohler and Milstein fused antibody-producing mouse spleen cells with mouse myeloma cells (Hurrell, 1988, and Roitt, 1994) The hybrid formed i e the fusion product, secretes antibodies of the specificity dictated by the spleen cells, of the immunised animal, but in the quantity characteristic of a myeloma An outline of the procedure for the production of monoclonal antibodies is shown in Figure 5 1 3 2 1 The plasma cells of the spleen, taken from an immunised animal, are fused with a myeloma cell line The characteristics for the ideal myeloma cell are

1 The myeloma cell does not secrete an antibody of its own

2 They are usually deficient in the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) HGPRT deficient cells when grown in media containing hypoxanthine, aminopterin and thymidine (HAT) cannot synthesis DNA

3 The cell has the necessary machinery needed for high level of protein synthesis and secretion

4 The cells are well characterised in terms of growth characteristics and genetics

The fusion products formed are isolated by selective growth on HAT medium In the HAT medium the myeloma cells die because they cannot synthesise DNA for their survival The spleen cells die naturally in culture In the HAT media the hybridoma can synthesise DNA and it will survive The hybridoma cells are not HGPRT deficient thanks to the spleen cell, and it will survive due to the robust nature of the myeloma One then has an immortalised hybridoma cell that produces antibodies Many hybridomas will be formed by the fusion of myeloma and the splenocytes and each in turn will produce an antibody One hybrid secretes one specific antibody The various antibodies secreted are screened by the use of enzyme-linked immuno-sorbent assay (ELISA), or radio immunoassays (RIA) The hybrid selectively secreting the antibody of interest is then cloned The cells are then produced in batch culture, or as ascitic tumours, and the antibody is purified from solution and characterised

Monoclonal antibodies are used in cancer diagnosis and therapy (Borrebaeck and Larrick, 1990, Epenetos, 1991, Roitt, 1994, and Kuby, 1994) including tumour imaging, in blood group analysis (Roitt 1994, and Kuby, 1994), in virology (McMichael and Fabre, 1982) They can be radiolabelled and used for the detection of tumours and metastases (Epenetos, 1991) They are used in affinity columns for isolation of interferons, growth factors, and tumour antigens (Kuby, 1994) Monoclonal antibodies can also be used for the removal of drugs from the circulation e g digoxin and addictive drugs (Hurrell, 1988) They can be used for targeting of drugs (e g ricin) or radioactive material to tumour sites for treatment of cancer (Roitt 1994, and Kuby 1994) These are only some of the numerous applications of monoclonal antibodies Table 5 1 3 1 is a comparison of some of the properties of monoclonal and polyclonal antibodies

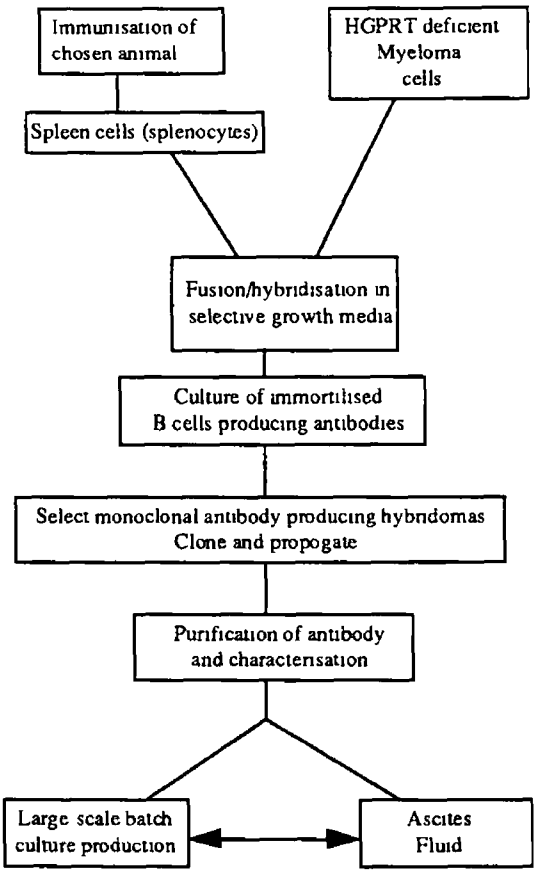


Figure 5 1 3 2 1 Scheme for the production of monoclonal antibodies (procedure described in text) The diagram is adapted from Cahill et al (1995)

5 1 3 3 Antibody engineering

One form of antibody production used involves the isolation of the genes that code for the production of heavy and/or light chains from cells of the immune system from the animal of interest (Killard *et al*, 1995) These cells can be from the spleen, blood, lymph node or bone marrow These genes can be combined and expressed as antibody proteins on the surface of a phage particle (a virus that infects bacterial cells) The phage producing the best antibody affinities are transfected into bacteria and the bacteria are grown in media. Antibodies produced by the transfected bacteria can be isolated from the media, or from cell lysate The antibodies are then purified and characterised Typically only portions of the antibodies e g V_H, V_L, and C_H or a combination of them

i e the Fab fragment (see Figure 5 1 1) are produced by antibody engineering The antibody fragments prepared can be used in sensor applications (Killard *et al* , 1995) or the system can be adapted to produce humanised antibodies Antibodies from a non-human foreign species will illicit an immune response in humans The humanised antibodies produced by antibody engineering will overcome this major disadvantage of antibodies for use in therapy or imaging in humans

5 1 3 4 Bifunctional antibodies

An antibody has two arms that bind to the same antigen (Figure 5 1 1) which function in triggering effector cells and in forming immune complexes Antibodies, that have binding specificities for two unique species, can be produced by several methods Bispecific/bifunctional antibodies have specificity for two distinct antigens (Nolan and O'Kennedy, 1992, Fanger, 1995, and Cahill *et al* , 1995) Figure 5 1 3 4 1 illustrates two of the methods available for the production of these antibodies

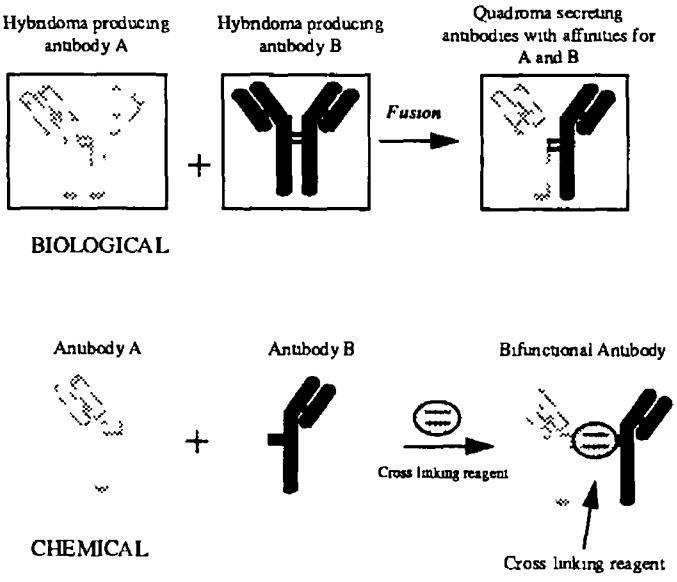


Figure 5 1 3 4 1 Strategies for the biological and chemical production of bispecific antibodies The biological production of bifunctional antibodies requires the fusion of two hybridomas, that produce two different antibodies A and B, to form a quadroma which secretes a bifunctional antibody with the same specificities as antibodies A and B Most chemical methods start with pepsin digestion to produce $F(ab')_2$ fragments (see Figure 5 1 1) of two unique antibodies The disulphide bonds are reduced to the F(ab') fragments, using mercaptoethylamine, in the presence of sodium arsenite and Ellman's agent The F(ab') fragments from each antibody are mixed in equimolar quantities and recombined under appropriate conditions to produce the hybrid $F(ab')_2$ bifunctional antibody

The bispecific nature of the bifunctional antibody lends them to uses in novel applications e g non-enzyme-based immuno assays, and in drug targeting to tumour sites The biological production of bifunctional antibodies involves the fusion of two hybridomas, producing two specific antibodies, to form a quadroma that secretes antibodies with a bispecific nature determined by the two specific antibodies Chemical techniques for the production of bifunctional antibodies typically start with pepsin digestion to produce $F(ab')_2$ fragments (see Figure 5 1 1) of two unique antibodies The disulphide bonds are reduced to produce the F(ab') fragments, using mercaptoethylamine, in the presence of sodium arsenite and Ellman's agent The F(ab') fragments from each antibody are mixed in equimolar quantities and recombined under appropriate (e g they can be recombined using o-phenylene dimaleimide as a cross-linking agent), conditions to produce the hybrid $F(ab')_2$ bifunctional antibody

Bifunctional antibodies can be used in non-enzyme-based immunoassays (see section 1 4 1 1 2) or in drug tumour therapy (Fanger, 1995) They have been used for the determination of 7-hydroxycoumarin in urine (see section 1 4 1 1 2) In drug tumour therapy one arm of a bifunctional antibody can be directed at tumour specific antigens and the other arm at a drug or toxin The antibody will bind to the drug or toxin while the other arm is free to find the tumour antigen and bind to it giving association site directed drug targeting

5 1.4. Detection systems for the measurement of antibody-antigen interactions

The techniques of radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are sensitive for detecting antigens and antibodies, and are extremely economical in the use of reagents (Roitt *et al* , 1989) RIA and ELISA are the most widely used immunological assays Multiple assays may be carried out in a relatively short time In RIA, detection is based on the measurement of the radioactivity emission from a radioactive label on an antibody or antigen The units of measurement are counts per minute (cpm) and it is related to the amount of radioactive species labelled to the

antigen or antibody present The assay system used in RIA is similar to that employed for ELISA (see section 5 1 4 1) In an ELISA assay system, detection is, typically, based on a colourimetric change after the reaction of an enzyme with its substrate to produce a coloured product The absorbance of the coloured product formed is dependent on the amount of antibody-antigen interaction in the assay The antibodies and antigens used in ELISA should be relatively pure to prevent non-specific reactions which may give high background readings Section 1 4 outlines the antibody-based immunoanalytical techniques for the determination of coumarin and 7-hydroxycoumarin Section 5 1 4 1 outlines the theory, advantages and disadvantages of an ELISA and it describes an ELISA for the determination of 7-hydroxycoumarin in urine

5 1 4 1 Enzyme-Linked Immunosorbent Assay (ELISA)

One of the most popular methods for measuring the antibody-antigen interaction is the ELISA Figure 5 1 4 1 1 shows the principle of the use of enzyme labels in a basic immunoassay Firstly, the antigen is immobilised to the surface of a plastic well of an ELISA plate (typically in a '96-well' format) An antibody (labelled with an enzyme) raised against the antigen is then added and allowed to interact with the immobilised antigen

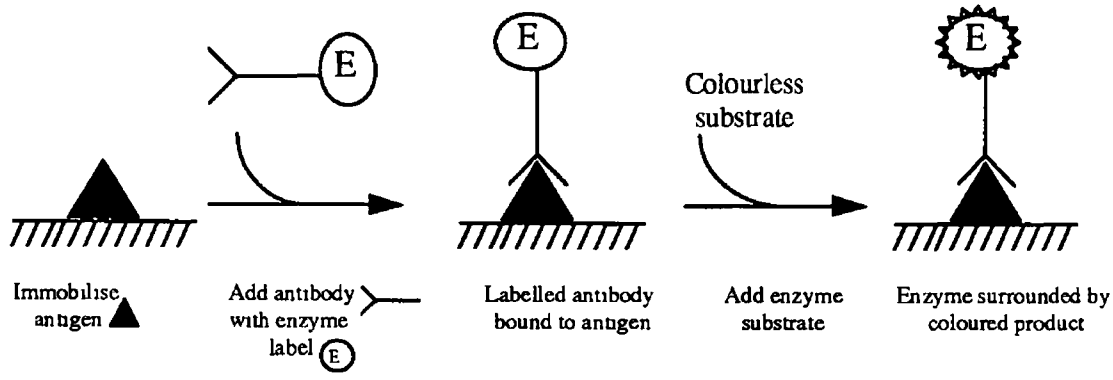


Figure 5 1 4 1 1 Schematic representation showing the basic principle in the use of enzyme labels in a basic immunoassay The presence of the antigen is detected using the enzyme-labelled antibody The amount of coloured product is proportional to the concentration of the enzyme and thus to the concentration of the antigen

A colourless substrate, for that particular enzyme, is then added and the amount of coloured product formed is determined spectrophotometrically The amount of coloured product is proportional to the amount of enzyme present and thus, to the concentration of the antigen All ELISA are adaptations of the one described in Figure 5 1 4 1 1 ELISA's can be adapted for each particular application Heterogenous (see section 1 4) ELISA's can be divided into competitive and non-competitive systems An example of a competitive ELISA, is where antibody is immobilised on a solid surface of a 96 well ELISA plate Enzyme-labelled and unlabelled antigen are then added into the assay well after washing A competition between the labelled and unlabelled antigen for antibody-antigen interaction occurs After the removal of unbound antigen, the substrate for the enzyme is added and the amount of coloured product, i e the absorbance, formed by the reaction of the enzyme and substrate is determined The enzyme activity (e g determined by the absorbance of the enzyme-substrate reaction) is inversely proportional to the concentration of unlabelled antigen

In non-competitive ELISA e g antibody is immobilised at the solid surface After washing away any antibody excess, antigen is added and allowed to bind to the antibody A secondary enzyme-labelled antibody (which reacts with a different epitope on the antigen) is then added and the enzyme activity is determined after the addition of the substrate The enzymes used in an ELISA must fill the following criteria They must be cheap, easy to conjugate chemically without loosing their activity, be stable, have high purity, be easily prepared, there should be no endogenous enzyme activity in the samples, no interfering species should be present that would inhibit enzyme activity or cause pH extremes, and the product formed should be easily detectable The most common enzymes and their respective substrates are given in Table 5 1 4 1 1

Enzyme	Substrate
Horseradish peroxidase	o-phenylenediamine
Alkaline phosphatase	p-nitrophenylphosphate
β-galactosidase	o-nitrophenylphosphate
Glucose oxidase	β-D-glucose
Urease	urea

Table 5 1 4 1 1 The enzymes and substrates most commonly used in ELISA (Table taken from Tijssen, 1985)

There are many advantages of enzyme immunoassays These include
(I) The assay is highly sensitivity and requires very small reagent volumes, (ii) The antigen-antibody interaction can be of a high specificity avoiding any interferences from cross-reactivity and interference from other endogenous species present, (iii) The assays are relatively cheap and relatively rapid, (iv) Both reproducible qualitative and quantitative results can be obtained, (v) The product formed between the enzyme and the substrate is, generally, easy to detect colourimetrically, (vi) Automation of the assay is straight forward, (vii) Enzyme immunoassays are very versatile and can be adapted for a variety of applications, (viii) The use of enzymes removes the need for radioactive labels and thus, removes problems associated with radiation, (ix) Enzyme immunoassays are applicable for use with monoclonal and polyclonal antibodies (see section 5 1 3)

The disadvantages and problems with enzyme immunoassays include
(I) The antigen-antibody interaction is dependent on the specificity of the antibody for the antigen For polyclonal antibodies, there can be batch to batch variations in specificities, (ii) Extraneous reactions may interfere and cause non-specific binding of antibodies to solid surfaces, cells etc This can be overcome by improving blocking procedures, (iii) It is important to ensure that the antibodies and enzymes used are still active and that they are stable in the reaction system, (iv) In order to detect some antigen-antibody interactions it is sometimes necessary to add further steps to amplify enzyme-substrate activity signal and increase sensitivity, (v) The antibody-enzyme labelling may interfere with enzyme activity and antibody-antigen binding The antigen-antibody binding and enzyme activities must be tested to ensure that no major alterations have occurred in binding characteristics, (vi) If the enzyme label used is on the antigen, it is necessary to ensure that there is successful binding of the label to the antigen This must be both in quantity and quality i e the enzyme activity and the antigenic site must be maintained

Enzyme immunoassays are widely used for a variety of applications (Braithwaite, 1995, Marks and Kwasowski, 1995, Fukal, 1995, Miyaishi, 1995) These include the detection and monitoring of drug levels e g amphetamines, benzodiazepines, and

morphine (Braithwaite, 1995, Marks and Kwasowski, 1995) They are used for measurement of protein levels associated with disease e g Factor VIII in haemophilia and for detection of hormone levels e g β-HCG in pregnancy (Marks and Kwasowski, 1995) They are used for the detection of food additives, food contaminants and food constituents, e g proteins, mycotoxins, and pesticides (Fukal, 1995) They are also applied to the area of forensic science e g bloodstain analysis, saliva, semen and drug level analysis (Miyaishi, 1995)

5 1 4 2 Determination of 7-hydroxycoumarin by an antigen-inhibition ELISA

The ELISA developed for the determination of 7-hydroxycoumarin was a competitive 'antigen-inhibition' ELISA (Reinartz *et al* , 1995) Figure 5 1 4 2 1 is a diagrammatic representation of the antigen-inhibition ELISA developed for the determination of 7-hydroxycoumarin (see section 2 5 4) It shows what occurs in one single well of a 96 well plate The assay uses an enzyme-labelled antibody raised against rabbit IgG (the source of the anti-7-hydroxycoumarin antibody) The following steps are the steps in the antigen-inhibition ELISA for the determination of 7-hydroxycoumarin (Figure 5 1 4 2 1)

Step 1 Immobilisation of bovine serum albumin (BSA)-7-hydroxycoumarin (BSA-7-OHC) protein-drug conjugate to the activated plate surface Non-covalent interactions, of the hydrophobic type, are the main interactions responsible for this adherence Unbound protein-drug is washed away with phosphate buffered saline (PBS)

Step 2 To prevent non-specific binding by other proteins e g anti-7- hydroxycoumarin antibody, or anti-rabbit-HRP-labelled, any remaining available sites on the plate surface are 'blocked' with BSA Unbound protein is removed with detergent (Tween-20) in PBS

Step 3 7-hydroxycoumarin standards or dilutions of the anti-7-hydroxycoumarin antibody are prepared in PBS A competition for the antibody occurs, between the 7-hydroxycoumarin coated, via the protein, on the plate and that of the free 7-

hydroxycoumarin in solution The amount of free 7- hydroxycoumarin will determine the amount of antibody available to bind to the coated 7- hydroxycoumarin

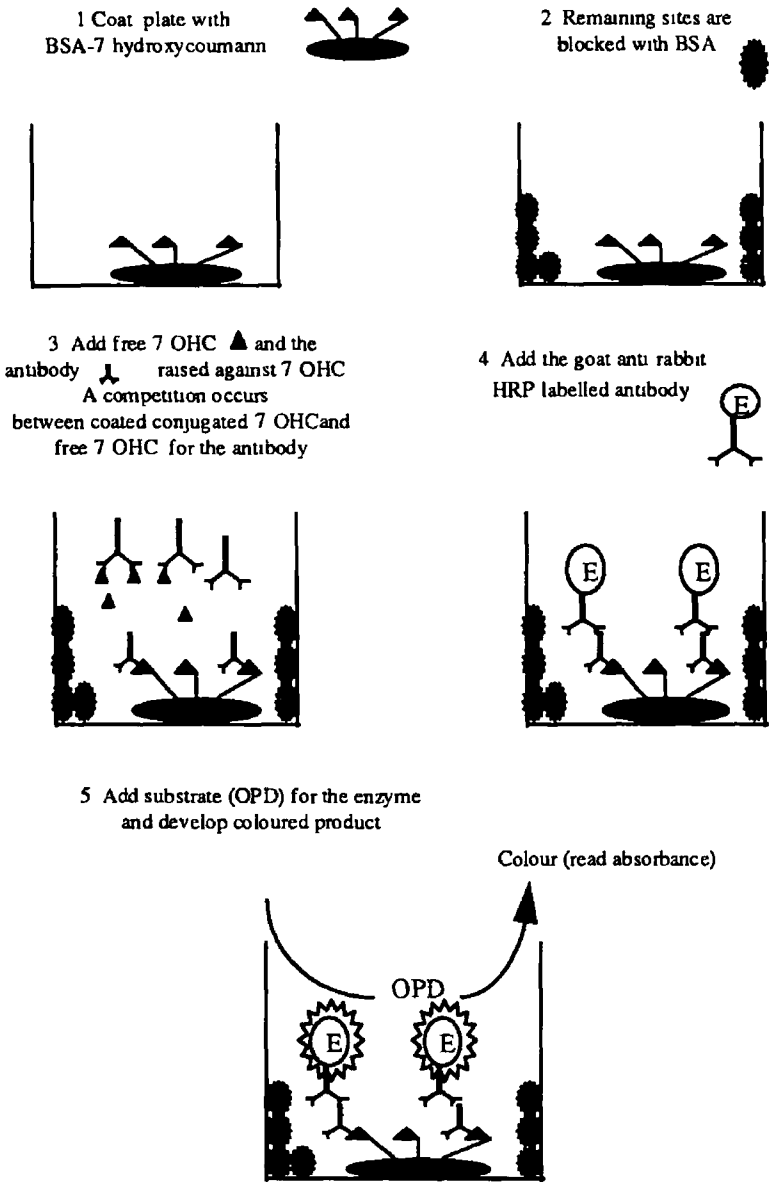


Figure 5 1 4 2 1 The principle steps in an antigen-inhibition ELISA for the determination of 7-hydroxycoumarin (7-OHC) by the use of anti-7-hydroxycoumarin antibodies The steps involved in the assay are detailed in the text

Thus, a relationship between the 7-hydroxycoumarin concentration and the amount of antibody at the plate surface is formed Unwanted antibody, free 7-hydroxycoumarin and antibody-7-hydroxycoumarin complexes are removed with washing

Step 4 Addition of a secondary antibody with an enzyme label It is antibody raised in a goat against rabbit IgG and labelled with horse-radish peroxidase (HRP) Following binding of the secondary antibody to the anti-7-hydroxycoumarin antibody and washing any excess away, the enzyme substrate is added (step 5)

Step 5 o-Phenylene diamine (OPD) reacts with horse-radish peroxidase (HRP) to give a yellow product (see Table 5 1 4 1 1) The absorbance of the resultant colour is related to the amount of free 7- hydroxycoumarin added in step 3 To calculate the antibody titre, at step 3 no 7- hydroxycoumarin is added However, dilutions of the antibody are prepared and added Using a 96 well plate it is possible to carry out multiple analysis Each well is an individual assay In theory it is possible to carry out 96 assays concurrently However, the wells are usually used for analysis in duplicate and the mean absorbance values are calculated for each standard or unknown

Step 6 The absorbance reading is proportional to the free 7-hydroxycoumarin concentration added The absorbance of the standards (A) were divided by the maximum absorbance value A_0 (A/A_0) and plotted versus the log 7-hydroxycoumarin concentration From this standard curve the concentrations of unknown 7-hydroxycoumarin concentrations may be calculated

5 2. PRODUCTION OF 7-HYDROXYCOUMARIN-PROTEIN CONJUGATES USED FOR IMMUNISATION TO PRODUCE ANTI-7-HYDROXYCOUMARIN ANTIBODIES AND FOR THE SCREENING OF THESE ANTIBODIES.

The conjugation of 7-hydroxycoumarin utilising haptenic aromatic amino group conversion to a diazonium group

It is necessary to conjugate 7-hydroxycoumarin to a carrier protein in order to stimulate the immune response of a host animal to produce antibodies against it Conjugation of 7-hydroxycoumarin to proteins is achieved by preparing the 3-amino-7-hydroxycoumarin derivative (see Figure 2 5 1 1 in section 2 5 1) The conjugation reaction occurs in two stages The reaction requires the diazotisation of the amino group on the hapten to diazonium chloride with HCl and $NaNO_2$, which then spontaneously couples with the histidine, tyrosine and tryptophan residues on the carrier protein molecule (see Figure 2 5 1 1 and section 2 5 1) [Catty and Raykundalia, 1988] The highly reactive diazonium ion (in alkaline solution) reacts with the aromatic ring of the amino acid residues on the protein The 7-hydroxycoumarin was conjugated to thyroglobulin and bovine serum albumin

The thyroglobulin-7-hydroxycoumarin produced was used in an immunisation protocol for the production of polyclonal sera in New Zealand White rabbits (see section 2 5 2) In order to monitor for the production of antibodies to 7-hydroxycoumarin (see sections 2 5 4 and 2 5 5) it was necessary to prepare another conjugate of 7-hydroxycoumarin It is not possible to directly immobilise 7-hydroxycoumarin to a plastic surface In order to monitor for a specific anti-7-hydroxycoumarin antibody from the general population of antibodies in the polyclonal antisera, a bovine serum albumin (**B**SA)-7-hydroxycoumarin conjugate was produced This was used for screening the antisera for the presence of anti-7-hydroxycoumarin antibodies in an ELISA (see section 5 1 4 2) The use of the thyroglobulin-7-hydroxycoumarin conjugate would give a very high background signal due to the presence of many antibodies in the antisera to many epitopes on the thyroglobuhn surface There is enough of a difference between the

surfaces of thyroglobulin and bovine serum albumin to reduce interferences due to any background signals from antibodies to epitopes on the bovine serum albumin surface

5 2.1. Synthesis and characterisation of 7-hydroxycoumarin derivatives

The reaction and procedure for the production of 7-hydroxycoumarin derivatives is described in section 2 5 1 (see also Figure 2 5 1 1) The method was that described by Egan (1993)

5 2 1 1 3-acetylamino-7-aceto-coumarin

The compound produced from the refluxing of 2,4-dihydroxybenzaldehyde, glycine, sodium acetate and acetic anhydride gave a single spot on TLC after washing and recrystallisation from glacial acetic acid The yield was 30 %, and the compound had a melting point of 238 °C (literature value - 234 °C) The infra red (IR) analysis (profile not shown) showed C=O stretch (1770 cm^{-1}, 1720 cm^{-1}, 1670 cm^{-1}), N-H stretch (3380 cm^{-1}) and C-H stretch (3100 cm^{-1}) It is difficult to assign all the other bands due to interference from carbonyl groups etc that may cause a shift in the stretching frequency

5 2 1 2 3-amino-7-hydroxycoumarin

The 3-acetylamino-7-aceto-coumarin was refluxed in hydrochloric acid to produce 3-amino-7-hydroxycoumarin After neutralisation, filtration and recrystallisation from water methanol the product was analysed by IR, nuclear magnetic resonance (NMR), and TLC Analysis of the compound produced by TLC gave two spots which corresponded to the TLC of a standard prepared by Egan (1993) which also had two spots under TLC The IR analysis (Figure 5 2 1 2 1) showed asymmetric and symmetric stretches of N-H bond (in NH_2 moiety) at 3450 cm^{-1}, and 3350 cm^{-1}), O-H stretch (3200 cm^{-1}) and C=O (1700 cm^{-1}) Speculative identification of other peaks is difficult due to shifts in frequencies caused by other groups present on the molecule e g carbonyl groups cause band shifts (James, personal communication, 1996)

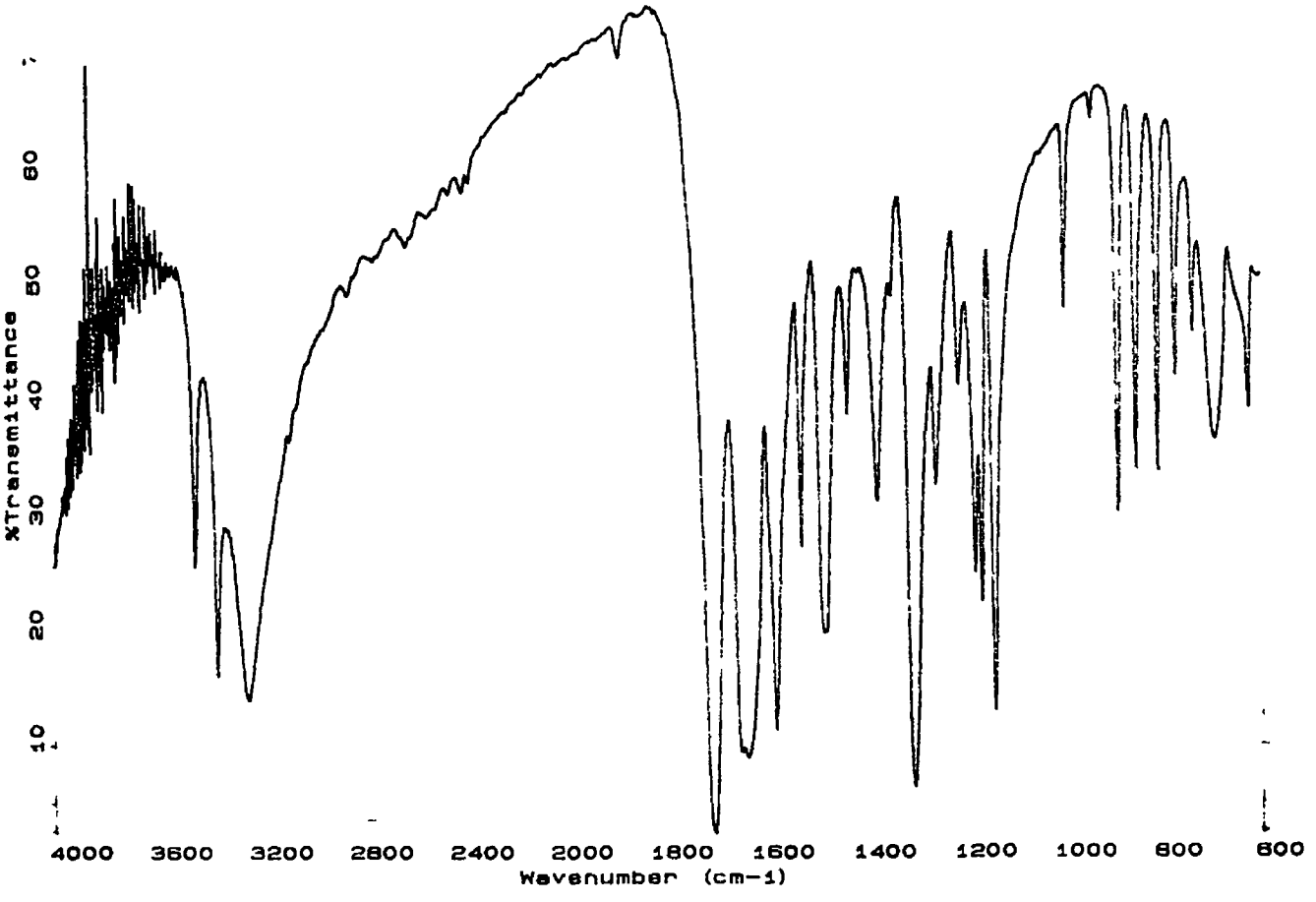


Figure 5 2 1 2 1 Infra red analysis of 3-amino-7-hydroxycoumarin The IR analysis (Figure 5 2 1.2 1) showed asymmetric and symmetric stretches of N-H bond (in NH_2) at 3450 cm^{-1}, and 3350 cm^{-1}), O-H stretch (3200 cm^{-1}) and C=O (1700 cm^{-1}) The infra red spectrometer used was a Nicolet I R Spectrometer The infra red spectra was scanned between 4000 cm^{-1} and 600 cm^{-1} and plotted against the % transmittance

Proton NMR analysis (Figure 5 2 1 2 3) of 3-amino-7-hydroxycoumarin (Figure 5 2 1 2 2) showed the following chemical shifts A singlet was seen at 5 3 ppm and it was assigned to NH_2 A doublet and two singlets are seen as a complex mixture at 6 7 ppm and they are assigned to the two singlet aromatic H's and one of the doublet aromatic H's The doublet at 7 1 ppm is due to the other doublet aromatic H's The peak at 10 ppm was due to the hydrogen in the hydroxy moiety The peaks at 2 1 ppm and 2 5 ppm were due to the dimethylsulphoxide The integration is approximately

1 1 3 2 which would indicate the OH, Aromatic C-H, 3 x aromatic C-H's, and NH_2 when related to the structure

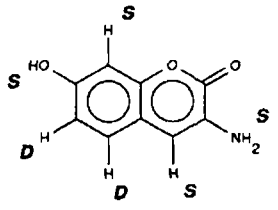


Figure 5 2 1.2 2 Structure of 3-amino-7-hydroxycoumarin The hydrogen groups and the multiplicity (i e a single splitting giving a doublet) observed for each hydrogen S indicates that there should be a singlet (single peak) and D, that there should be a doublet in the NMR spectrum

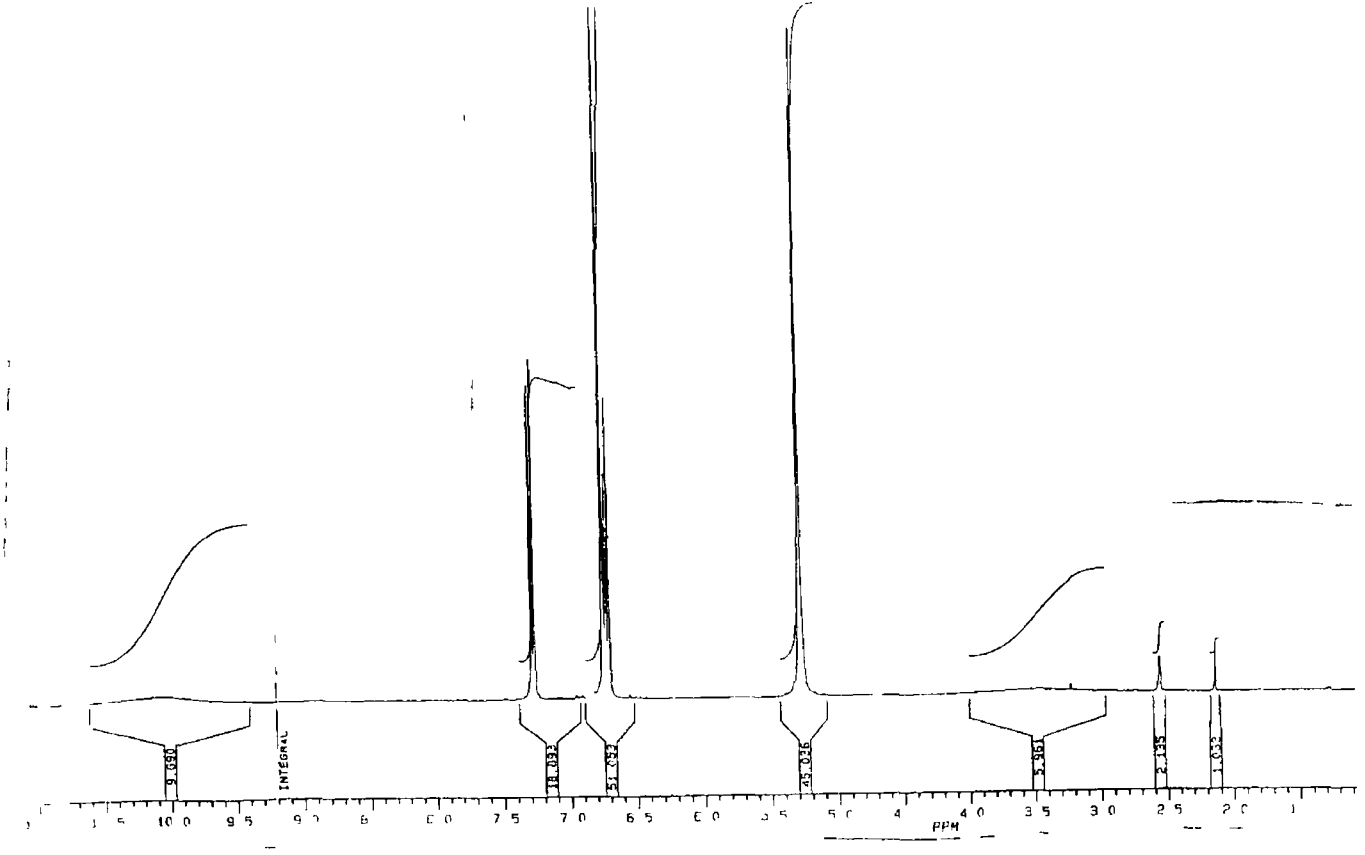


Figure 5 2 1.2 3 Nuclear magnetic resonance spectrum of 3-amino-7-hydroxycoumarin Proton NMR analysis of 3-amino-7-hydroxycoumarin (Figure 5 2 1 2 2) showed the following chemical shifts A singlet was seen at 5 3 ppm and it was assigned to NH_2 A doublet and two singlets are seen as a complex mixture at 6 7 ppm and they are assigned to the two singlet aromatic H's and one of the doublet aromatic H's The doublet at 7 1 ppm is due to the other doublet aromatic H's The peak at 10 ppm was due to the hydrogen in the hydroxy moiety The peaks at 2 1 ppm and 2 5 ppm were due to the dimethylsulphoxide The integration is approximately 1 1 3 2 which would indicate the OH, Aromatic C-H, 3 x aromatic C-H's, and NH_2 when related to the structure The nuclear magnetic resonance spectrometer used was a Bruker 400 MHz.

5 2 1 3 Characterisation of thyroglobulin-7-hydroxycoumarin and bovine serum albumin-7-hydroxycoumarin conjugates

5 2 1 3 1 Ultraviolet (UV) spectral analysis

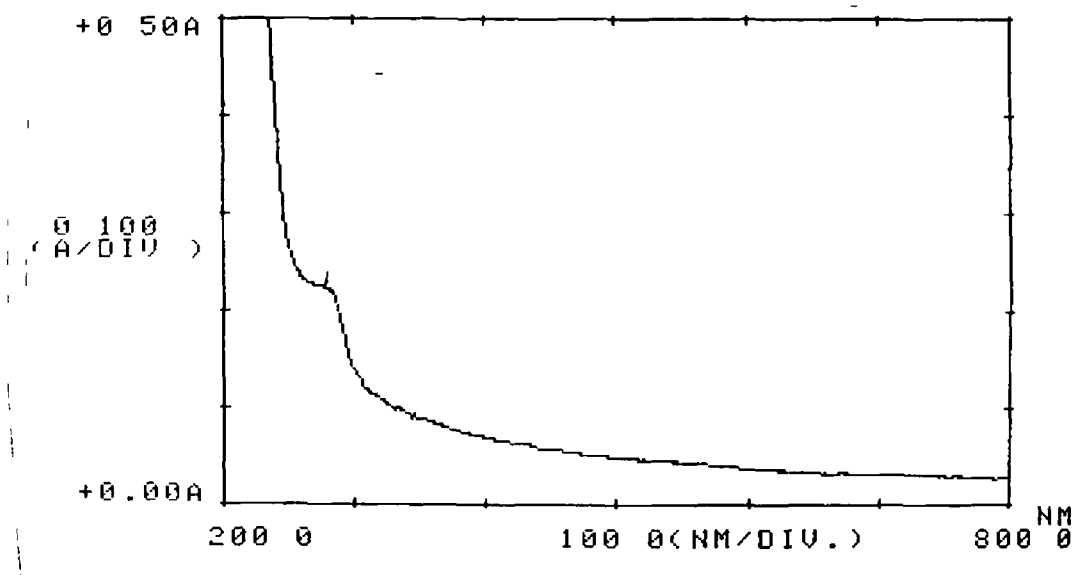
Control thyroglobulin (see section 2 5 1) and the thyroglobulin-7-hydroxycoumarin conjugate, formed by the diazotisation of 3-amino-7-hydroxycoumarin and its subsequent conjugation to a carrier protein (see section 2 5 1) were prepared in ultra pure water and scanned between 200 nm and 800 nm on a UV spectrophotometer Figures 5 2 1 3 1 (a) and (b) show the UV spectra of control thyroglobulin and thyroglobulin-7-hydroxycoumarin, respectively Control thyroglobulin had absorbance maxima at 200 nm and at 280 nm The brown compound formed had absorbance maxima at 200 nm, 280 nm, 330 nm and 480 nm Compounds with azo moieties are generally coloured, as observed (absorbances in the visible region) with the protein-drug conjugate produced The control thyroglobulin was not coloured (no peak absorbances in the visible region) The results for the BSA were similar (results not shown) From the UV spectra analysis it was shown that a new compound had been formed that was very different from the starting material The spectra was also different to that of 7-hydroxycoumarin which has absorbance maxima at 324 nm and 210 nm (spectra not shown)

5 2 1 3 2 Analysis of conjugates by gel filtration -Sephadex G25 column

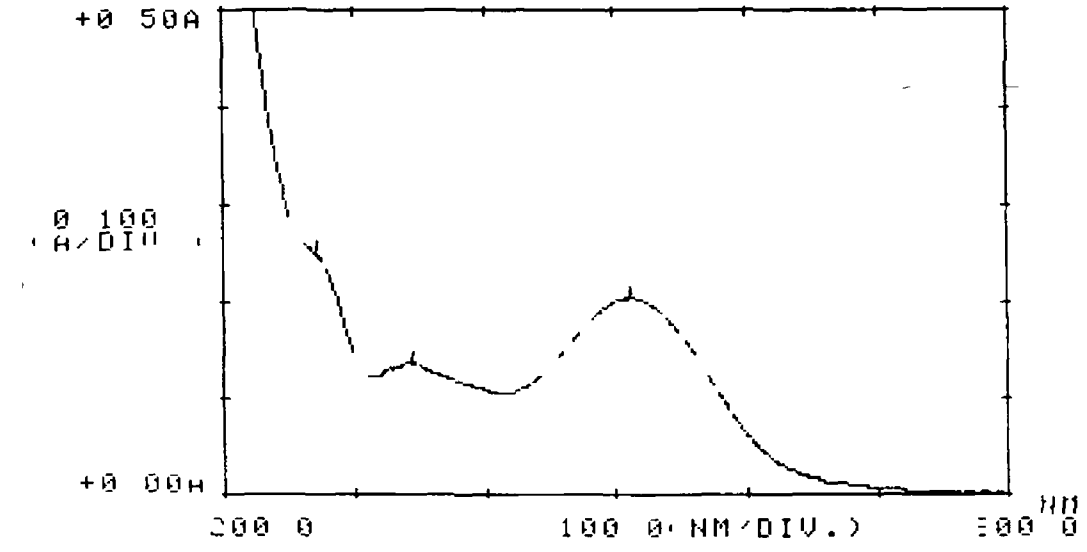
A 9 cm X 1 cm Sephadex G25 (Size range 86-258 μm, fractionation range 100 to 5000 Da, and the useful pH range was pH 2-13) column was prepared and allowed to equilibrate in ultra-pure water The fractionation range of the Sephadex was 100 to 5000 molecular weight units Thus, any free 7-hydroxycoumarin or unconjugated 3-amino-7-hydroxycoumarin should not elute in the void volume All species with molecular weights above 5000 Da should elute in the void volume Blue dextran (molecular weight ca 2,000,000 Da) was used to determine the void volume of the column The thyroglobulin-7-hydroxycoumarin, prepared in water, was then applied and the brown band migrated in the void volume indicating that the coloured compound was of a large molecular weight The UV scan of the eluting coloured compound was recorded (results not shown) and it was found to be the same as that shown in Figure

5 2 1 3 1 i e for thyroglobulin-7-hydroxycoumarin No coloured species was observed at the top of the Sephadex column indicating that none of the reaction mixture, which was a red/brown colour was present after the reaction and dialysis and that the band was due only to a large molecular weight species Control thyroglobulin also eluted in the void volume (determined by UV analysis of eluted fractions)

(a)



(b)



Figures 5 2 1 3 1 (a) and (b) show the UV spectra of control thyroglobulin and thyroglobulin-7-hydroxycoumarin. The spectrum were taken between 200 nm and 800 nm and show peak absorbances at 200 nm, and 280 nm for the control thyroglobulin, and at 200 nm, 280 nm, 330 nm and 480 nm for the thyroglobulin-7-hydroxycoumarin

5.2.1.3.3. Analysis of the conjugates by HPLC

A method was developed for the separation of thyroglobulin and bovine serum albumin by HPLC on a Protein-Pak column SW 300 (see section 2.2.3.3.) with UV detection at 280 nm and analysis of the compounds was also carried out with photodiode array detection. It was impossible to differentiate the two molecular weights for thyroglobulin and thyroglobulin-7-hydroxycoumarin peaks based on their retention times. The protein-pak acts separates species based on their respective molecular weights. Both control thyroglobulin and thyroglobulin-7-hydroxycoumarin had retention times of 8.9 min ± 0.02 min. Figure 5.2.1.3.2. shows the overlay of the spectra obtained at four different retention times for control thyroglobulin. It is a plot of wavelength (nm) versus absorbance (A.U.).

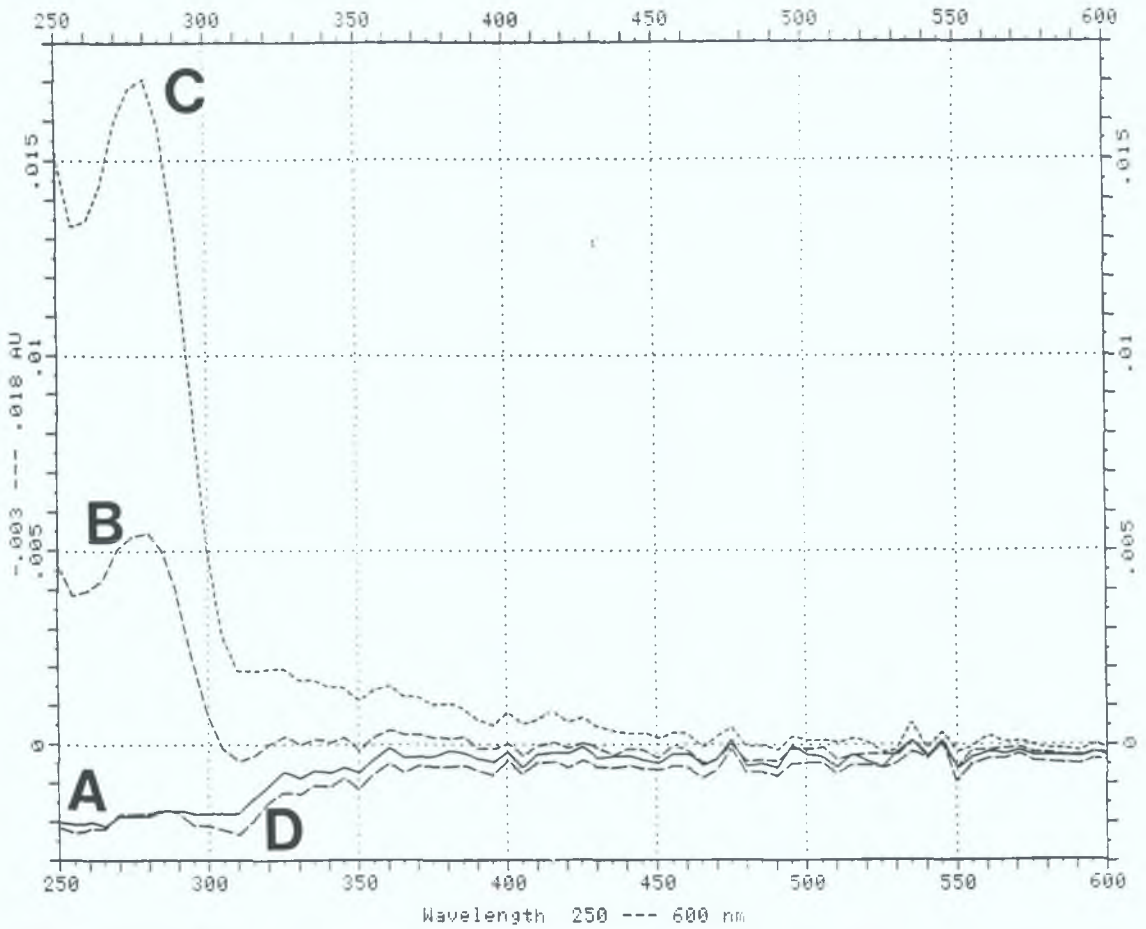


Figure 5.2.1.3.2. Overlay of the spectra obtained at four different retention times for control thyroglobulin. The sample was analysed by HPLC with a 100 % ultra-pure water mobile phase (flow 0.5 ml/min) with photodiode array detection. (5 nm resolution). The spectra were taken at the following times (A) 7.5 min, (B) 8.4 min, (C) 9.0 min, and (D) 10.3 min, respectively.

The times are 7 4 min, 8 4 min, 8 9 min and 10 3 min, respectively The times correspond to the chromatogram where (A) there are no other peaks, (B) the side of the main peak, (C) the crest of the main peak and (D) after the chromatogram has returned to the baseline Figure 5 2 1 3 3 shows the overlay of the spectra obtained at four different retention times for thyroglobulin-7-hydroxycoumarin The times of each of the spectra are the same as in Figure 5 1 2 3 2

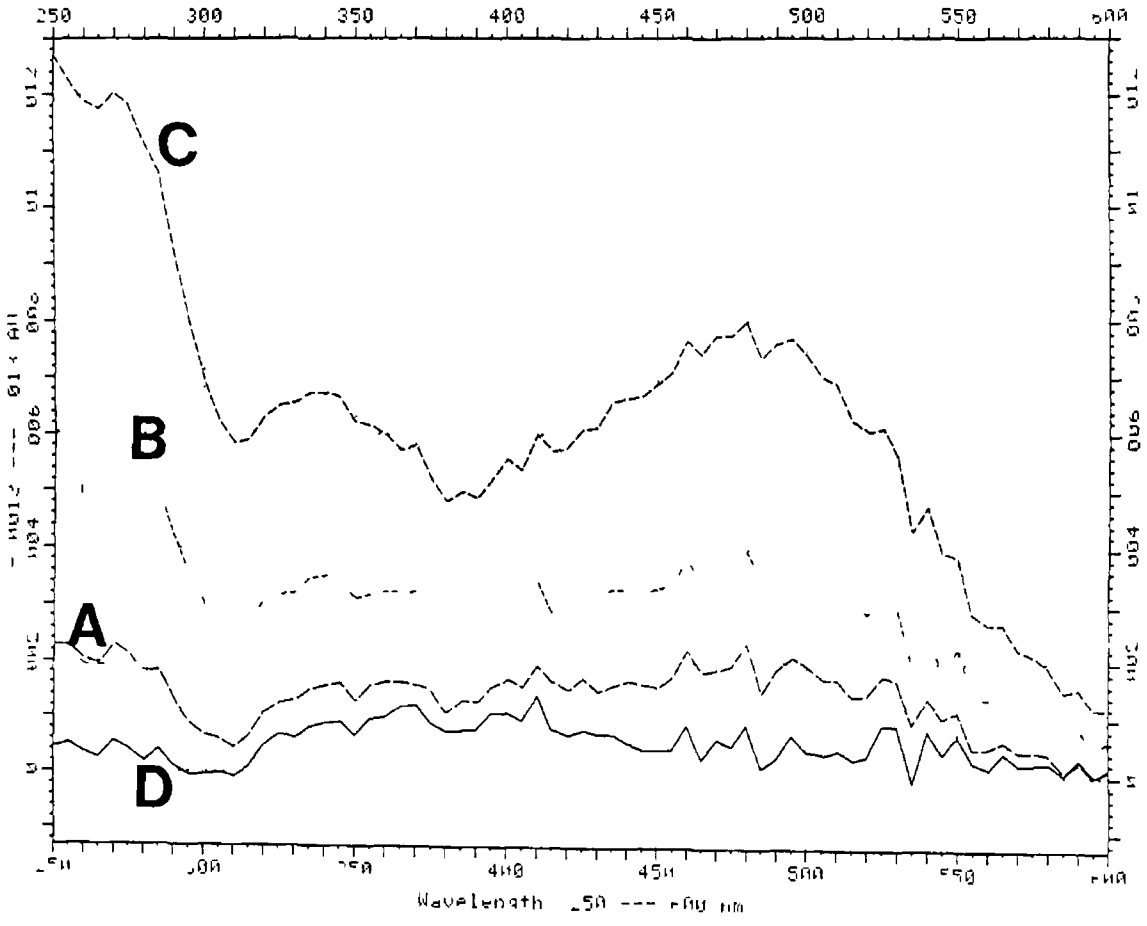


Figure 5 1 2 3 3 Overlay of the spectra obtained at four different retention times for thyroglobulin-7-hydroxycoumarin The sample was analysed by HPLC with a 100 % ultra-pure water mobile phase (flow 0 5 ml/min) with photodiode array detection (5 nm resolution) The spectra were taken at the following times (A) 8 0 min, (B) 8 6 min, (C) 9 0 min, and (D) 10 3 min, respectively

It is clearly seen that the compound produced by the diazocoupling of the 3-amino-7-hydroxycoumarin to thyroglobulin gives a very different profile to that of the control thyroglobulin Thus, it was concluded that a new species had been produced that was as a result of the diazotisation of 7-hydroxycoumarin to thyroglobulin, and similarly for bovine serum albumin (results not shown) A plot of contour profile of time versus wavelength for the diazocoupled thyroglobulin-7-hydroxycoumarin is shown in Figure 5 2 1 3 4 The peak was scanned from 250 nm to 600 nm from 7 min to 12 min The peak maxima are seen at 480 nm, 330 nm and 280 nm

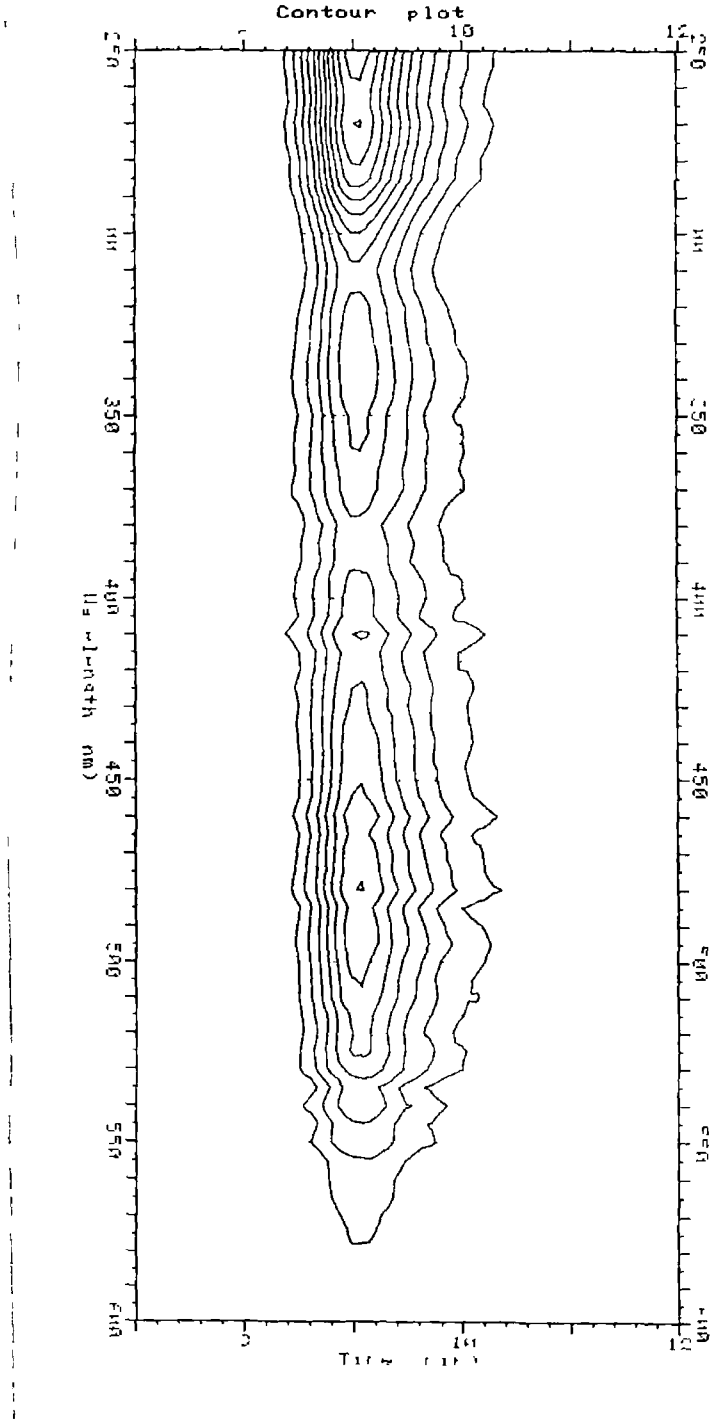


Figure 5 2 1 3 4 A plot of contour profile of time versus wavelength for the diazocoupled thyroglobulin-7-hydroxycoumarin is shown in Figure 5 2 1 3 4 The peak was scanned from 250 nm to 600 nm from 7 min to 12 min . The peak maxima are seen at 480 nm, 330 nm and 280 nm. The sample was analysed by HPLC on a Protein pak SW 300 with a 100 % ultra-pure water mobile phase (flow 0 5 ml/min) with photodiode array detection (5 nm resolution)

5 3 PRODUCTION AND PURIFICATION OF ANTI-7-HYDROXYCOUMARIN ANTIBODIES

5 3 1 Purification of anti-7-hydroxycoumarin antibodies from serum

IgG's are the most basic of the proteins in serum and they have properties that allow their separation from other proteins on the basis of their different physicochemical properties e g salt fractionation, and affinity chromatography (Tijssen, 1985) The anti-7-hydroxycoumarin antisera was purified by ammonium sulphate precipitation and Affi-T affinity chromatography (see section 2 5 3) Reinartz *et al* , (1995) described a method for the affinity purification of only anti-7-hydroxycoumarin antibodies Precipitation by salting-out to remove non-specific proteins is highly effective, simple, cheap, it avoids direct effects on the protein and concentrates the protein After precipitation of the IgG the sample was reconstituted into the running buffer before separation and purification by affinity chromatography

Affi-T is a thiophilic agent that binds the thiol- groups present in IgG, thus, allowing a separation of all IgG present It does not selectively separate out anti-7-hydroxycoumarin antibodies The column was allowed to equilibrate in running buffer (0 75 M $(NH_4)_2SO_4$) and the sample was then applied to the column and fractions were taken every 1 ml The absorbance of every fraction was monitored at 280 nm Figure 5 3 1 1 shows a plot of the fraction number (1 ml fractions) versus absorbance (A U , monitored at 280 nm) for the purification of the polyclonal antisera by Affi-T affinity chromatography after ammonium sulphate precipitation When the absorbance had decreased to 0 1 A U (absorbance units) the running buffer was replaced with the elution buffer (50 mM tris-HCl, pH 7 4) at the point marked in Figure 5 3 1 1 The fractions (25-30) with the highest absorbances were pooled and dialysed against 0 1 M PBS, pH 7 4 at 4 °C The concentration of the resultant compound in solution was determined by the Bicinchoninic acid (BCA) assay, and characterised by HPLC to assess its molecular weight (see section 5 3 2) and purity

The antibody titre (section 5 3 3) and cross reactivity (section 5 3 4) was determined for the purified antibody After characterisation it was used in an ELISA for the determination of free and total 7-hydroxycoumarin in urine samples from a volunteer who had been treated with coumarin

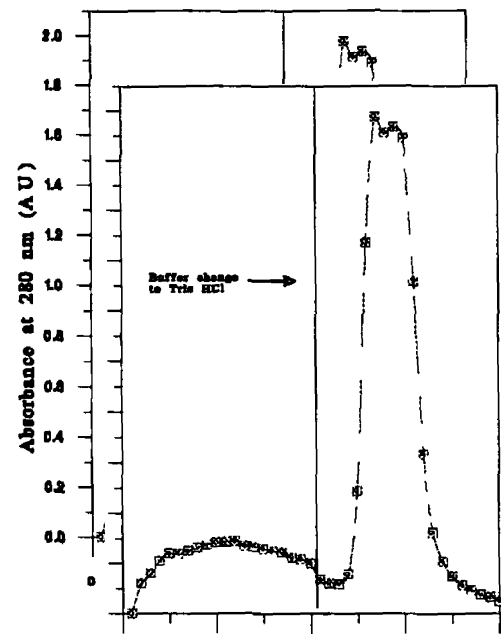


Figure 5 3 1 1 Plot of fraction no (1 ml fractions) versus absorbance at 280 nm (A U) for the purification of anti-7-hydroxycoumarin antibodies by Affi-T affinity chromatography When the absorbance had decreased to 0 1 A U (absorbance units) the running buffer was replaced (fraction 21) with the elution buffer (50 mM tris-HCl, pH 7 4) The fractions with the highest absorbances (25-30) were pooled and dialysed against PBS The method is described in section 2 5 3

5 3 2 Analysis of an anti-7-hydroxycoumarin antibody by HPLC

High-performance liquid chromatography was used to assess the purity of the antibodies purified after affinity chromatography The molecular weight of the antibody was also determined by this method by comparing the retention times of the antibody to those of a set of protein molecular weight standards Figure 5 3 2 1 shows a chromatogram of the separation of the purified antibody Purity was determined from a comparison of the percentage area occupied by the peak of interest to all other peaks present This is

determined by System Gold software (see section 4 0) The purity of the antibody in this sample was 97 5 %

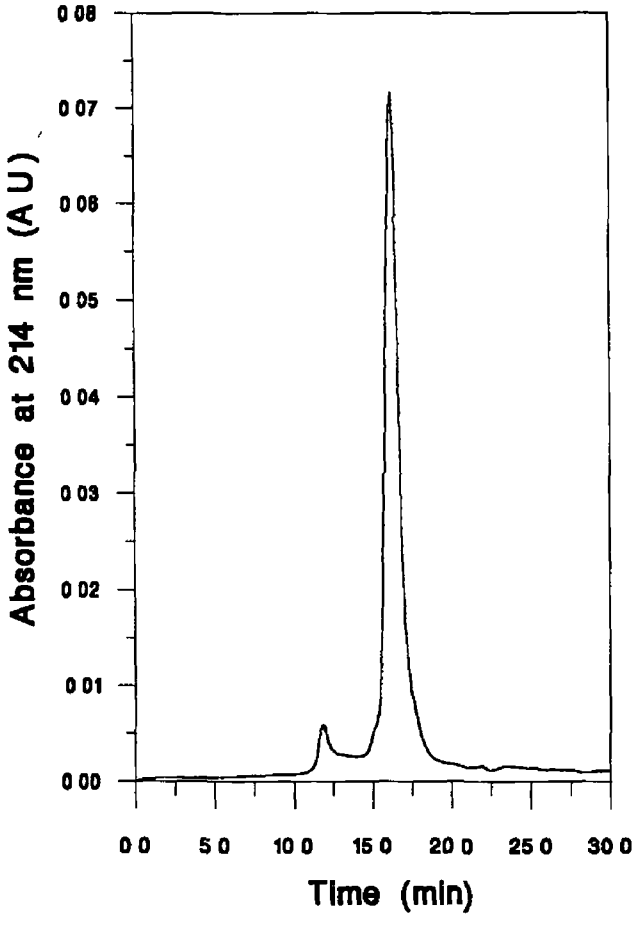


Figure 5 3.2 1 Chromatogram of the separation of an antibody purified by salt precipitation and affinity chromatography The sample was analysed by HPLC as described in section 2 2 3 3 The retention time was 16 2 min and the purity of the peak was 97 5 % Separation was carried out on a Protein-Pak SW 300 (particle diameter 10 µm) The flow rate was 0 5 ml/min and detection was at 280 nm

The molecular weight of the antibody was determined from a plot of the log of the molecular weight versus retention times for a set of protein standards / retention time of an unretained protein i e blue dextran (Figure 5 3 2 2) The equation of the line was Y = 3 03 - 0 31 Log X, R^2 = 0 98 The molecular weight protein standards analysed, their molecular weights and their respective retention times were- cytochrome c (ca 12,400

Da) 20 7 mın, carbonic anhydrase (ca 29,000 Da) 19 5 mın, bovine serum albumin (ca 66,000 Da) 17 7 mın, β-amylase (ca 200,000 Da) 15 9 mın, apoferritin (ca 443,000 Da) 15 3 mın and blue dextran (ca 2,000,000 Da) was 11 5 mın The retention time for the antibody was 16 2 mın The molecular weight determined for the antibody was ca 159,300 Da

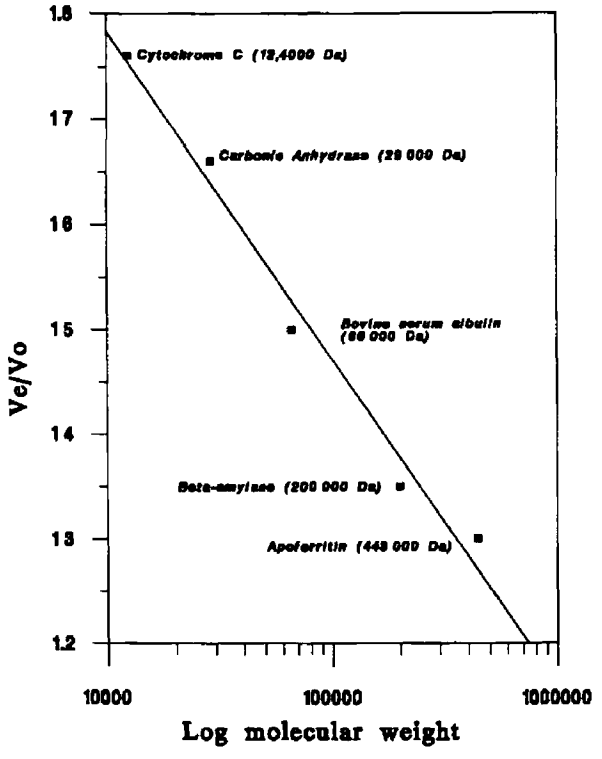


Figure 5 3 2 2 Plot of the log molecular weight of protein standards versus retention time The proteins analysed, their molecular weights and their respective retention times were- cytochrome c (ca. 12,400 Da) 20 7 mın, carbonic anhydrase (ca 29,000 Da) 19 5 mın, bovine serum albumin (ca 66,000 Da) 17 7 mın, , β-amylase (ca 200,000 Da) 15 9 mın, apoferritin (ca 443,000 Da) 15 3 mın and blue dextran (ca 2,000,000 Da) was 11 5 mın The graph was used for the determination of the molecular weight of the purified anti-7-hydroxycoumarin antibody The retention time for the antibody was 16 2 mın The molecular weight of the antibody was determined as ca. 159,300 The equation of the line was y = 3 03 - 0 31 log X, R^2 = 0 96 The samples were analysed by HPLC as described in section 2 2 3 3

5 3 3. Determination of antibody activity (titre)

The antiserum and purified antibody were serially diluted (in 1 % (w/v) BSA in 0 14 M PBS, pH 7 4 containing 0 05 % (v/v) tween-20) and the activity of the antibody was

assessed at the different dilutions (Table 5 3 3 1) This was tested by preparing the ELISA plate as described in section 2 5 4 A range of antisera and antibody dilutions were prepared (1/500 - 1/160,000) and these were added to each of six wells No free 7-hydroxycoumarin was added The secondary antibody was then added and the colour was developed as described in section 2 5 4 Table 5 3 3 1 are the results for the mean absorbances (n=6) ± standard deviation, for the antisera and the purified antibody and the respective dilutions prepared

Dilution	Absorbance at 405 nm (A U) Antibody analysis in unpurified antisera	Absorbance at 405 nm (A U) Antibody analysis after purification
1/500		1 01 ± 8E-3
1/1000		0 74 ± 0 02
1/1250	0 90 ± 0 02	
1/2000		0 42 ± 0 02
1/2500	0 75 ± 0 01	
1/4000		0 25 ± 0 01
1/5000	0 58 ± 0 02	
1/8000		0 18 ± 0 01
1/10000	0 39 ± 0 02	
1/16000		0 12 ± 0 01
1/20000	0 26 ± 0 01	
1/32000		0 09 ± 4E-3
1/40000	0 17 ± 0 01	
1/64000		0 07 ± 1E-3
1/80000	0 11 ± 0 01	
1/128000		0 05 ± 2E-3
1/160000	0 09 ± 4E-3	
Negative control	0 07 ± 2E-3	0 05 ± 2E-3

Table 5 3 3 1 Table of the mean absorbances ± standard deviation (n=6) as determined by ELISA (see section 2 5 4) and the dilutions of rabbit antisera and purified antibody These absorbances were used to determine the titre of the antibody The negative control was carried out as described in section 2 5 4 except there was no bovine serum albumin-7-hydroxycoumarin coated on the plate The results were plotted against the log of the reciprocal dilution (see Figure 5 3 3 1)

The mean absorbances were plotted against the log of the reciprocal dilution (Figure 5 3 3 1) The ELISA on the negative control was carried out as described in section

2 5 4 except there was no BSA-7-OHC coated on the plate The remaining steps of the ELISA were carried out as described in section 2 5 4 For Figure 5 3 3 1 the negative control, i e the background reading, absorbance was subtracted from each mean absorbance

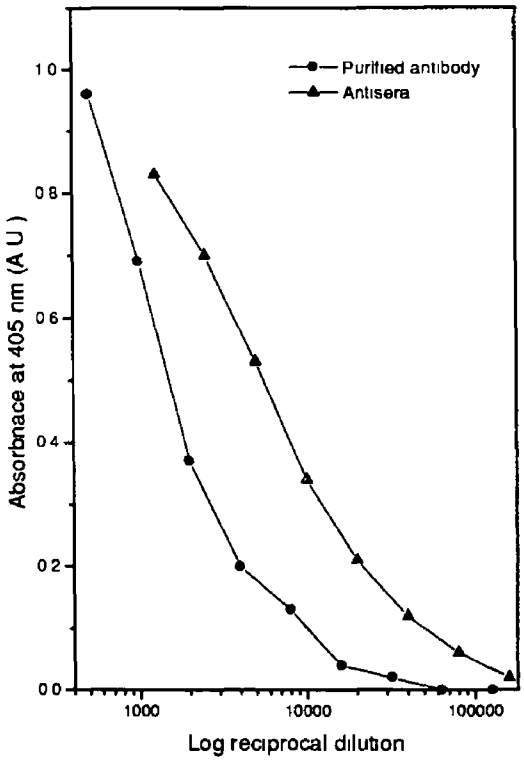


Figure 5 3 3 1 Plot of log reciprocal dilution versus absorbance at 405 nm (A U) for determination of antibody activity (titre) in the antisera and after purification The ELISA was carried out as described in section 2 5 4 The dilutions and their respective dilutions are tabulated in Table 5 3 3 1

Activity of the anti-7-hydroxycoumarin before purification from the serum i e in the antisera, was detectable to a dilution of 1/160,000 The activity of the antibody after purification was detected to a dilution of 1/64,000 The concentration of antibody used was 3 mg/ml At a dilution of 1/64,000 there was 47 picograms (4 7E-8 grams) of protein present per millilitre A dilution of 1/500 was used for the determination of 7-hydroxycoumarin in urine

5 3 4 Cross-reactivity of the anti-7-hydroxycoumarin antibody

The cross-reactivity of the purified antibody was assessed using an ELISA system as described in section 2 5 4 using several structurally related coumarin compounds instead of 7-hydroxycoumarin These were coumarin, coumarin-3-carboxylic acid, 7-hydroxycoumarin-glucuronide and 3-amino-7-hydroxycoumarin A range of 7-hydroxycoumarin concentrations (0 -25 µg/ml) were prepared and an ELISA was carried out (see section 2 5 4) The results from this ELISA was compared to the results from the ELISA carried out with the other coumarins The ELISA was carried out as described in section 2 5 4 The method was adapted by adding a set amount (10 µg/ml, 20 µg/ml or 25 µg/ml) of the compound, being assessed for the antibody cross-reactivity, as well as 7-hydroxycoumarin to each of three wells In other wells a range of concentrations (0, 1, 5, 10 and 20 µg/ml) of the compound under examination were added without any 7-hydroxycoumarin The absorbances after the addition of substrate were compared to the control ELISA results The antibody showed no cross-reactivity for coumarin, coumarin 3-carboxylic acid, or 7-hydroxycoumarin-glucuronide but there was some cross-reactivity for 3-amino-7-hydroxycoumarin (Figure 5 3 4 1) The activity of the antibody was not inhibited by coumarin, 7-hydroxycoumarin-glucuronide or by coumarin-3-carboxylic acid

Cross-reaction will occur for an antibody to a species with one identical determinant (see section 5 1 2 4) The other determinants will not have any affinity for the antigenic site The protein-conjugate produced, (see section 5 2) for immunisation of the rabbit host, involved the preparation of 3-amino-7-hydroxycoumarin (see section 5 2 1 2) This was then diazocoupled to thyroglobulin before its use in immunisation of the host species The structure of 3-amino-7-hydroxycoumarin is very similar (see section 5 1 2 4) to that of 7-hydroxycoumarin (Figure 5 3 4 2) i e it will possess similar determinants to that of the 7-hydroxycoumarin The 7-hydroxycoumarin moiety on the protein surface is held in the 3-position through the diazo coupling of the amino group to the aromatic amino acids (see section 5 2)

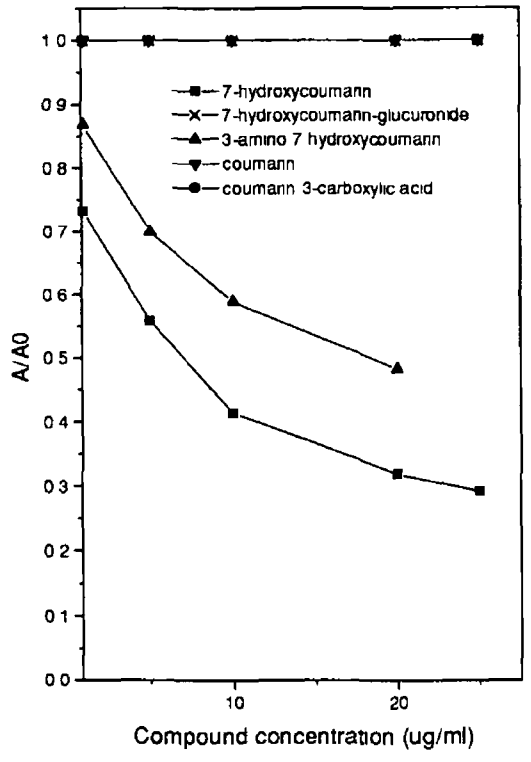


Figure 5 3 4 1 Assessing the cross-reactivity of the anti-7-hydroxycoumarin antibody The figure is a plot of concentration (μg/ml) of the compounds under examination versus A/A_0 There was some cross-reactivity with the 3-amino-7-hydroxycoumarin The legend for each compound is given on the plot

7-hydroxycoumarin 3-amino-7-hydroxycoumarin

Figure 5 3 4 2 Structures of 7-hydroxycoumarin and 3-amino-7-hydroxycoumarin

Only the 7-hydroxycoumarin moiety on the molecule is 'free' to interact with the immune system and an antibody will be raised against this determinant The 3-amino-moiety of the diazocoupled protein-drug complex cannot elicit an antibody response due to its positioning on the protein-drug conjugate Thus, the cross-reactivity observed for

3-amino-7-hydroxycoumarin was attributed to the 7-hydroxycoumarin side of the molecule

5.3 5 The use of the anti-7-hydroxycoumarin antibody for the determination of 7-hydroxycoumarin

The purified antibody was utilised in an ELISA for the determination of free and total 7-hydroxycoumarin in urine (see section 5 1 4 1 and 5 3 5 1) It was also used by Deasy *et al* , (1994) and Lu *et al* , (1996) for the determination of free 7-hydroxycoumarin using electrochemical detection An enzyme-linked electrochemical competitive immunoassay was developed by Deasy *et al* (1994) and is described in section 1 4 2 Lu *et al* (1996) developed a regeneratable enzyme immunosensor for 7-hydroxycoumarin based on electrochemical detection (see section 1 4 2) The methods and applications, utilising the purified antibody, are described A bispecific antibody was produced from the anti-thyroglobulin-7-hydroxycoumarin and an anti-alkaline phosphatase antibody (see section 1 4 1 1) The product was used to develop a one-step antigen inhibition ELISA for the detection of 7-hydroxycoumarin in urine (Reinartz *et al*, 1996) A competitive immunoassay for the analysis of 7-hydroxycoumarin in biological samples was developed, based on a commercial biosensor system (see section 1 4 3) - the BIAcore™ from Pharmacia Biosensor (Keating and O'Kennedy, 1995)

5 3 5 1 An ELISA for the determination of free and total 7-hydroxycoumarin in urine

The purified antibody was utilised in an ELISA for the determination of free and total 7-hydroxycoumarin in urine in a volunteer who had been treated with 250 mg of coumarin (see section 5 1 4 1 for a description of the ELISA, and see section 2 5 4 for the method) Samples and 7-hydroxycoumarin standards were prepared (see section 2 5 4) and analysed by the ELISA method as described by Reinartz et al , (1995) The optimum dilution of the purified antibody prepared for the ELISA was found to be 1/500 The samples prepared were also analysed by HPLC and by CE 10 µl of sample was taken for analysis by ELISA (see section 2 5 4), 20 µl of sample was taken for CE analysis (see section 2 3 3 1) and the remainder was used for HPLC analysis (adapted

from the gradient elution method described in section 2 2 2 2 2 - the sample was analysed after clean-up without the trichloroacetic acid step and without the addition of 4-hydroxycoumarin)

The mean absorbances (A) for each of the standards were calculated and divided by the maximal absorbance (A_0) [i e A/A_0] and plotted against the log of the concentration of 7-hydroxycoumarin (Figure 5 3 5 1) giving a standard curve from which the concentrations of 7-hydroxycoumarin in the unknown urine samples were determined

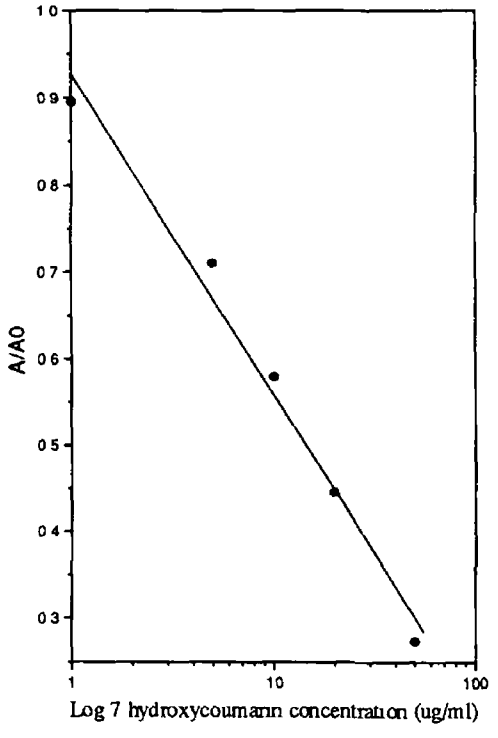


Figure 5 3 5 1 Plot of the log total 7-hydroxycoumarin concentration (0 - 50 µg/ml) versus the mean absorbances (A) divided by the maximal absorbance (A_0) [i e A/A_0] as determined by ELISA The 7-hydroxycoumarin was prepared in urine, extracted, reconstituted and analysed by ELISA as described in section 2 5 4 The equation of the line is $Y = 0\ 92 - 0\ 37 \log X$, $R^2 = 0\ 98$ The antibody dilution used in the ELISA was 1/500

The concentrations of free and total 7-hydroxycoumarin determined by each of the methods are given in Table 5 3 5 1 There was no statistical difference between each of the methods

Sample and Time	ELISA	CE method 1	HPLC
(hr)	7-hydroxycoumarin concentration (μg/ml)	7-hydroxycoumarin concentration (μg/ml)	7-hydroxycoumarin concentration (μg/ml)
Total 7-hydroxycoumarin			
0	3 0	2 0	0
2	1882 0	1980 0	1808 0
6	550 4	576 0	560 0
10	60 0	74 8	65 6
14	23 1	24 2	21 5
24	3 3	5 5	3 8
Free 7-hydroxycoumarin			
0	0	0	0
2	58 2	52 1	51 8
6	19 6	19 4	36 1
10	1 5	2 1	7 9
14	0	0	1 0
24	0	0	0

Table 5 3 5 1 Results for the determination of free and total 7-hydroxycoumarin in urine The samples were extracted into diethyl ether, evaporated to dryness and reconstituted into 200 μl of 25 mM phosphate buffer 10 μl of sample was taken for analysis by ELISA (see section 2 5 4), 20 μl of sample was taken for CE analysis (see section 2 3 3 1) and the remainder was used for HPLC analysis (adapted from the gradient elution method described in section 2 2 2 2 2 - the sample was analysed after clean-up without the trichloroacetic acid step and without the addition of 4-hydroxycoumarin) The results for the ELISA were determined from the mean absorbance A/A_0 (n=5) The CE and HPLC results were determined by single analysis

Summary

This chapter described the preparation of 7-hydroxycoumarin-protein conjugates by the diazocoupling of the 3-amino-7-hydroxycoumarin derivative to the carrier protein molecule i e thyroglobulin and bovine serum albumin These conjugates were characterised and the thyroglobulin conjugate was used for the immunisation of two rabbits for the production of anti-7-hydroxycoumarin antibodies The antibodies produced were screened with bovine serum albumin-7-hydroxycoumarin protein conjugate The antibodies were characterised and used for the determination of free and total 7-hydroxycoumarin in urine They were also used by other researchers for the preparation of a bispecific antibody (anti-7-hydroxycoumarin - anti-alkaline phosphatase), in the development of electrochemical immunosensors and in an assay on a commercial biosensor The conjugates produced and the resultant antibodies were shown to be very adaptable and useful for a range of applications

CHAPTER 6

OVERALL CONCLUSIONS

The work presented in this thesis has focused on three areas of the novel determination of coumarin, its metabolites, and their applications Figure 6 1 is a list of the some of the advantages and disadvantages of each method

Chapter 3 described the development and validation of several capillary electrophoretic separation methods for the determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide for a range of applications 7-hydroxycoumarin was extracted from urine and serum, as described before (Egan and O'Kennedy, 1992) but the analysis time was greatly decreased as compared to HPLC The method developed for the determination of 7-hydroxycoumarin in urine and serum was then adapted for the direct determination of 7-hydroxycoumarin after the *in vitro* metabolism of coumarin by liver microsomes in a range of different species The method was shown to be fast and reliable without the need for stopping the reaction before analysis Multiple runs were possible without any visible deterioration in capillary performance A separation method was then developed using another phosphate buffer-based electrolyte solution This was applied to the direct determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine without any sample clean-up and also to the study of the *in vitro* uridine diphosphate glucuronyl transferase activity in liver homogenate The method developed was both fast and reliable and required minimal sample preparation The methods developed and described in this chapter were applied to both *in vivo* and *in vitro* analysis of coumarin and 7-hydroxycoumarin metabolism

The CE methods developed were shown to be very applicable to these studies and comparable to any other analytical methods available Separations are fast, reproducibility is very good but the methods were found not to be as sensitive as HPLC or antibody detection CE is an excellent tool for the determination of coumarins However, with the development of fluorescence and electrochemical detectors the limits of sensitivity will, no doubt, make the method very sensitive for low level coumarin determinations Future ideas for developing CE for the analysis of coumarin may be in the area of determining inter-organ differences in UDPGT and β-glucuronidase activities The use of laser induced fluorescence (LIF) detection may be developed for the determination of the fluorescent 7-hydroxycoumarin Other *in vivo* and *in vitro* coumarin metabolites may be studied including 7-hydroxycoumarin-sulphate and 3-hydroxycoumarin

Chapter 4 outlined the development of a HPLC method for the direct determination of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine, plasma and serum with minimal sample preparation The HPLC separation method was validated in each of the biological fluids Samples of urine and plasma were obtained from volunteers who had been treated with coumarin and serum samples were obtained from patients who were treated with 7-hydroxycoumarin The method was also applied to the study of UDPGT activity in bovine liver homogenate The second section utilised the method of Egan and O'Kennedy (1992) for the study of the metabolism of 7-hydroxycoumarin in patients with advanced malignancies The serum drug profiles of 59 patients who had been treated with a range of doses of 7-hydroxycoumarin were determined HPLC was found to be a very adaptable technique for a range of applications with very good reproducibility and sensitivity The system was found to be very suitable for large scale determination of coumarin and some of its metabolites in serum, plasma and drug levels The method developed based on gradient elution had not been developed when the serum samples from the study were analysed This newer method would have been much less time consuming and less costly with respect to sample preparation and materials used Future studies for the use of HPLC in the analysis of coumarins may be to look at other coumarin metabolites both *in vivo* and *in vitro* in different organs and in different species

7-hydroxycoumarin-protein conjugates were prepared and utilised in the preparation of anti-7-hydroxycoumarin antibodies The methods of preparation and the characterisation and utilisation of the antibody is described in Chapter 5 The chapter describes the production of 7-hydroxycoumarin-thyroglobulin by the conjugation of 7-hydroxycoumarin utilising haptenic aromatic amino group conversion to a diazonium group The characterisation of the product formed and the components produced during each of the steps are detailed The conjugate was then used for immunisation to produce an anti-7-hydroxycoumarin antibody The antibody was purified, characterised and utilised for the determination of 7-hydroxycoumarin in urine by ELISA The antibody produced was also used by other researchers for the production of bispecific antibodies and their use in an antigen-inhibition ELISA, in the development of electrochemical immunosensors, and in an assay on a commercial biosensor The antibody used for the ELISA developed was sensitive for the determination of 7-hydroxycoumarin in urine The antibody was found to be cross-reactive for 3-amino-7-hydroxycoumarin The antibody was also found to be adaptable for a range of applications (i e production of the bispecific antibody etc) apart from its use unaltered i e in a basic ELISA The conjugates produced have also been used for the immunisation of mouse for the production of monoclonal antibodies and for the screening of antibodies made by genetic engineering and by *in vitro* immunisation The methods described for the preparation of each of the protein-7-hydroxycoumarin conjugates can be adapted for a range of proteins or for other 7-hydroxycoumarin derivatives The limit of other applications for the antibody was never reached It is possible to develop some other ELISA methods, to utilise antibody fragments for treatment and for the elucidation of 7-hydroxycoumarin mode of action It may also be possible to develop a novel assay on capillary electrophoresis to investigate the antibody-antigen interaction

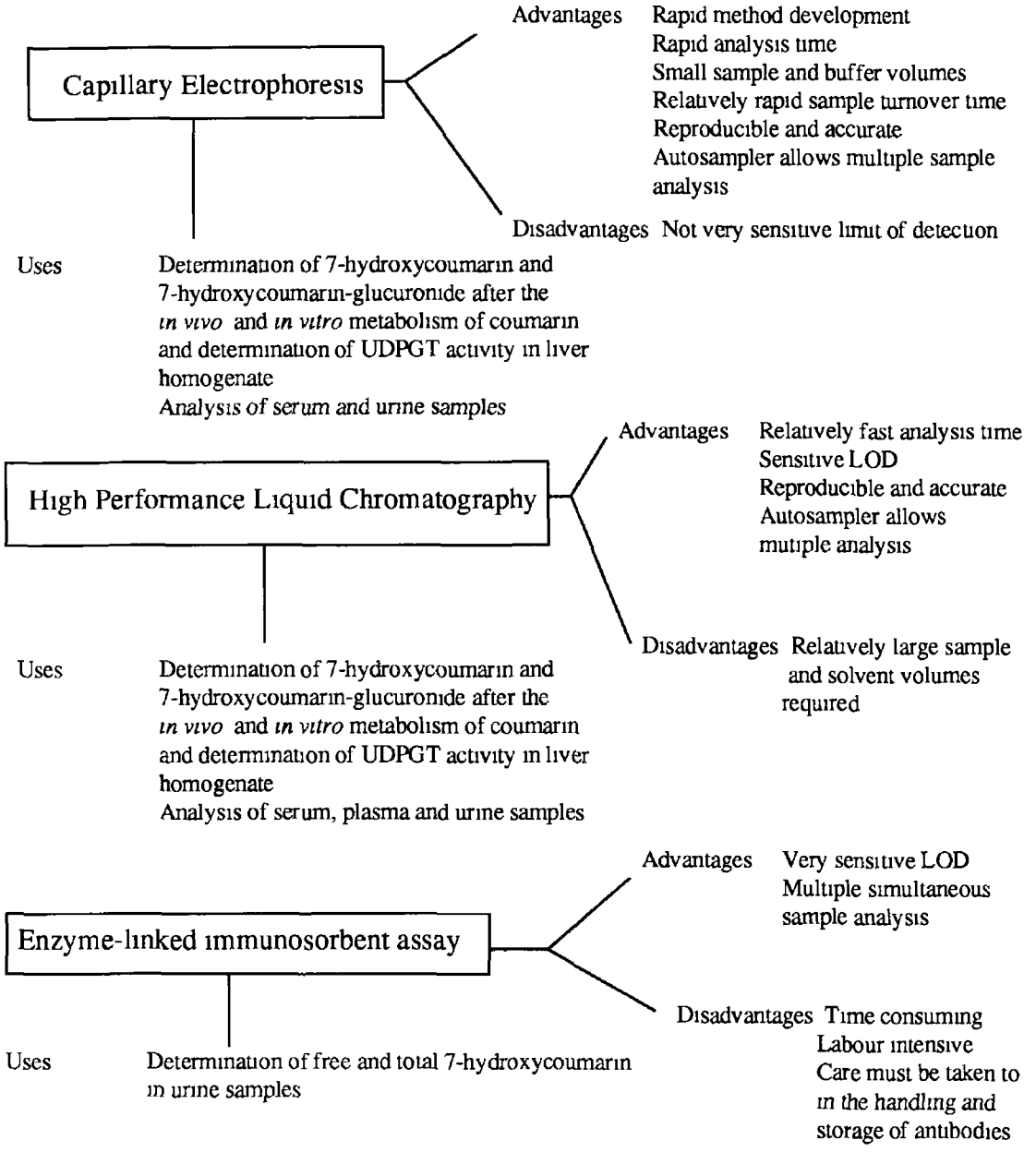


Figure 6 1 ***Advantages, disadvantages and uses of capillary electrophoresis, high performance liquid chromatography and an enzyme-linked immunosorbent assay***

CHAPTER 7

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